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DIPLOMA THESIS

RACK1 AS A CANDIDATE PROTEIN INVOLVED IN THE REGULATION OF TRANSLOCATION OF LCK TO LIPID RAFTS

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I hereby declare that I have written this thesis independently under supervision of RNDr. Dominik Filipp, Csc. in the laboratory of Immunobiology at Institute of Molecular Genetics AS CR v.v.i, with the use of listed literature.

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ABSTRACT

Two Src family tyrosine kinases Lck and Fyn are critical for the proximal T-cell signalling. Previously it was demonstrated that antibody-induced Lck activation outside lipid rafts (LR) results in Lck translocation to LR. Central in this event is the rapid translocation of kinase active Lck to LR, yet the mechanism underpinning this process is unknown. The main aim of this study is the characterization of molecular mechanisms and its functional elements regulating the early recruitment of signalling molecules to LR and forming immunological synapse. We have recently characterized the C-terminal YQPQP sequence as a novel *cis*-acting component essential for Lck partitioning to LR. In this context, the expression of the C-terminal truncate of constitutively active Lck (ΔFQPQP) in NIH3T3 cells failed to phosphorylate several proteins detected in the presence of untruncated kinase active Y505F-Lck. Comparative 2-D gel analyses followed by MS/MALDI identified RACK1 as a candidate protein for interaction with the C-terminal tail of Lck. Co-expression in NIH3T3 cells of RACK1 with either a wild type Lck or constitutively active Y505F-Lck revealed the complex formation between Y505F-Lck, but not WT-Lck, and RACK1. Ectopic expression of Y505F-Lck with its domain-inactivating mutations showed that Lck-RACK1 interaction depends on functional SH2 and SH3 domains, and the C-terminal tail sequence of Lck. Importantly, Lck-RACK1 interaction is readily detectable also in primary $CD4^+$ lymph node T-cells. Upon their activation, only the pool of Lck associated with the high molecular weight complexes translocates to lipid rafts. Co-purification of RACK1 with these fractions further suggests that it plays a role in the translocation of Lck to LR. This RACK1 function is further strengthened by observations that Lck and RACK1 co-redistribute to both antibody-mediated capping clusters and forming immunological synapses. Moreover, the importance of interaction between activated Lck and RACK1 in the context of Lck translocation mechanism to LR is further supported by data pointing to RACK1 association with elements of microtubular network. In aggregate, these results are the first to characterize RACK1 as a candidate molecule involved in the regulation of Lck translocation to LR by providing the link between Lck and cytoskeletal network.

Key Words: Lck, RACK1, Lipid Raft, T-cell activation, $CD4^+$ T-cell, Translocation

ABSTRAKT (CZ)

Dvě kinázy z rodiny Src, Lck a Fyn, jsou kriticky důležité pro zahájení časné T-buněčné signalizace. Předchozí experimenty prokázály, že aktivace kinázy Lck nacházející se mimo lipidové rafty (LR) vede k jejímu rychlému přesunu do těchto struktur. Mechanismus vysvětlující tento proces není dosud znám. Hlavním cílem této práce je charakterizace molekulárního mechanismu a jeho funkčních elementů, které se podílejí na regulaci časného přesunu signálních molekul do LR a vytváření imunologické synapse. Nedávno jsme charakterizovali C-terminální YQPQP sekvenci Lck jako novou komponentu důležitou pro lokalizaci Lck do LR. Bylo prokázáno, že odstranění C-terminálního konce konstitutivně aktivní Lck (ΔFQPQP-Lck) v NIH3T3 buňkách znemožňuje fosforylaci řady proteinů, které jsou detekovány v přítomnosti nezkrácené aktivní kinázy Y505F-Lck. Komparativní analýzou tohoto rozdílného fosforylačního statutu pomocí 2D-gelové elektroforézy a následné MS/MALDI byl identifikován protein RACK1 jako vhodný kandidát pro interakci s C-terminální částí Lck. Společná exprese proteinu RACK1 s divým typem Lck (WT-Lck) anebo konstitutivně aktivní formou Y505F-Lck v NIH3T3 buňkách prokázala formování komplexu mezi RACK1 a Y505F-Lck, ale ne s WT-Lck. Exprese Y505F-Lck s inaktivujícími mutacemi ukázala, že Lck-RACK1 interakce zavisí na přítomnosti funkčních domén SH2, SH3 a C-terminální sekvence Lck. Interakci Lck-RACK1 je také možno detekovat v primárních $CD4^+$ T-buňkách izolovaných z lymfatických uzlin. Po jejich aktivaci, pouze ta část Lck molekul, které jsou asociovany s vysokomolekulárními komplexy se může přemístit do LR. Přítomnost RACK1 ve stejných frakcích poukazuje na to, že tento protein může hrát úlohu v translokaci Lck do LR. Navíc Lck i RACK1 se společně přesouvají do formující se imunologické synapse i protilátkami indukovaných klastrů. Důležitost interakcí mezi aktivovanou Lck a RACK1 v kontextu tohoto translokačního mechanismu podtrhuje též zjištění, že RACK1 je asociován i s mikrotubulárním cytoskeletem. Ve shrnutí, tyto výsledky jako první popisují protein RACK1 jako kandidátní molekulu účastnící se regulace přesunu kinázy Lck do LR prostřednictvím napojení Lck na cytoskeletární síť.

Klíčová slova: Lck, RACK1, Lipidové Rafty, T-buněčná signalizace, $CD4^+$ T-lymfocyt, Translokace

Content

List of Abreviations

aa	*amino acid*
Ab	*antibody*
APC	*Antigen presenting cell*
BMDC	*Bone marrow derived dendritic cells*
BMMF	*Bone marrow derived macrophages*
Csk	*C-terminal src tyrosine kinase*
DAG	*diacylglycerol*
DC	*Dendritic cell*
DNA	*Deoxyribonucleic acid*
DPC	*Distal pole complex*
DRM	*Detergent resistant membrane*
ERM	*Ezrin radixin moesin family*
FAK	*Focal adhesion kinase*
FLNA	*Filamin A*
GEF	*Guanine nucleotide exchange factor*
GPI	*Glycosylphosphatidylinositol*
GST	*Glutathion S-transferase*
HA	*Hemagglutinin*
HMWF	*high molecular weight fraction*
IP_3	*inositol-1,4,5-triphosphate*
IS	*Immunological synapse*
ITAM	*Immunnoreceptor tyrosine-based activation motif*
ITIM	*Immunnoreceptor tyrosine-based inhibition motif*
kDa	*kilodalton*
LAT	*Linker for activated T-cell*
Lck	*Lymphocyte-specific protein tyrosine kinase*
LR	*Lipid raft*
MHCI	*Major histocompatibility complex glycoprotein class I*

MTN	*Microtubulin network*
MTOC	*Microtubule-organizing center*
O/N	*overnight*
OVA	*Ovalbumin*
PAG	*Phosphoprotein associated with Glycosphingolipid-enriched microdomains*
PFA	*paraformaldehyde*
PH	*Pleckstrin homology*
PIP_2	*phosphatidylinositol biphosphate*
PKC	*Protein kinase C*
PLCγ	*Phosphopolipase C gamma*
PM	*Plasma membrane*
PMA	*phorbol 12-myristate 13-acetate*
PTK	*Protein tyrosine kinase*
PTP	*Protein tyrosine phosphatase*
RACK1	*Receptor for activateted C kinase*
RNA	*Ribonucleic acid*
SFK	*Src family kinase*
SGFA	*sucrose gradient floatation assay*
SH2	*Src homology 2*
SH3	*Src homology 3*
SLP-76	*SH2 domain containing leukocyte protein of 76 kDa*
SMAC	*Supramolecular activation cluster*
TCR	*T-cell receptor*
WB	*Western blotting*
ZAP-70	*ξ-chain associated protein of 70kDa*

1 Introduction

Immunity, a vital component of life systems, is a very important part of our defence against various threats of danger, either self- or non-self derived. It protects us from pathogen attacks, environment changes, genetic disorders etc., but sometimes it also can be harmful for ourselves. We tend to divide immune responses into two modules – *innate*, non-specific and *adaptive*, or acquired. Both components actively cooperate and communicate with each other via direct cell-cell interactions or indirectly through a release of cytokine and chemokine mediators and thus creating a highly integrated and coordinated network of communication channels.

This study is focused on T lymphocytes, also called T-cells that play a fundamental role in adaptive immunity. There are two major subsets of T-cells: $CD4^+$ helper T-cells and $CD8^+$ cytotoxic T-cells, both being able to recognize antigens presented on the surface of Antigen Presenting Cells (APC) in the context of MHC class II and MHC class I molecules, respectively. An antigen-mediated T-cell:APC interaction leads to the initiation of T-cell activation and triggering of downstream signalling pathways, resulting in the global changes in gene expression. Despite a controversy surrounding the physiological and structural heterogeneity of plasma membrane (Munro, 2003), the involvement of membrane microdomains called Lipid Rafts (LR) in T-cell activation is well established (Horejsi, 2003). In this context, LR form very dynamic membrane clusters that contain a set of important signalling molecules and thus provide the platform for their membrane distribution in non-activated T-cells. Importantly, LR have been shown to be also involved in the regulation of spatio-temporal redistribution of signalling molecules during the onset of T-cell activation. It has been suggested that these early membrane translocation events are controlled by cytoskeleton network (Viola and Gupta, 2007). However, the mechanism linking the T-cell Receptor (TCR) engagement with the initiation of cytoskeleton reorganization remains largely unknown.

Two Src Family tyrosine Kinases (SFK) Lck and Fyn are critical for the proximal T-cell signalling. It has been previously demonstrated that antibody-induced Lck activation outside LR results in Lck translocation to LR (Filipp, 2003). Central in this event is the rapid translocation of kinase active Lck to LR. Thus, the main aim of our study is the characterization of molecular mechanism(s) and its functional elements regulating the early recruitment of signalling molecules to LR and forming immunological synapse.

Using the fibroblast model system, we previously identified several candidate proteins potentially involved in the process of Lck membrane distribution. Here, we report the first characterization of one of these candidate proteins, called RACK1, as the molecule involved in regulation of translocation of Lck to LR through linking the Lck kinase to cytoskeletal network.

2 Literature Review

This section details the current view on T-cell activation process with emphasis on the initiation of TCR signalling and activation of critically important signalling cascades. The model presented describes various molecular components of these signalling pathways, including TCR receptor, CD4 and CD28 co-receptors and two Src-family kinases Lck and Fyn, mechanism of their interactions, and their specific roles in signal transduction process. Further, it highlights the induction of cytoskeletal reorganization and changes in gene expression as the consequence of these initial signalling events. It also characterizes LR as very important structural components of plasma membrane for the initiation of T-cell signalling. Further, the relationships between these three major players (signalling molecules, LR, cytoskeleton) that together orchestrate the highly regulated process of T-cell activation leading to the formation of Immunological Synapse (IS) and ultimately resulting in full activation of effector T-cells, is reviewed. At the end, we briefly describe the structure and function of the protein called Receptor for Activated C Kinase 1 (RACK1). This molecule discovered some time ago has been shown to serve as a scaffold protein for several isoforms of Protein Kinase C (PKC) as well as for SFKs. Data presented in this study identified RACK1 protein as an important cytoskeletal element supporting the translocation of Lck kinase to LR in early phases of T-cell activation.

2.1 Activation of T-cells: two signals required

T-cell activation begins by TCR-mediated recognition of specific antigens presented on APCs, usually dendritic cells (DC), macrophages or B-cells. Antigens are always

presented in the context of the Major Histocompatibility Complex glycoproteins class I or II (MHCI or MHCII) in a form of short peptide fragments, usually 10-20 amino acids long. They can be of self- or non-self origin (endo- or exo-genous, respectively). Upon recognition of peptide-MHC complexes (pMHC), TCR molecules expressed on the surface of T-cells, bind to pMHC complexes and this binding is considered to be an initiation point (foundation-stone) for the formation of IS (see below). Beside this interaction which provides the first activation signal for T-cells, the second, so called co-stimulatory signal is also required for full T-cell activation (Lenschow et al., 1996). Co-stimulation is provided by interactions between B7-family members expressed on APCs (B7.1/CD80 and/or B7.2/CD86) and their ligand CD28 present on T-cells.

2.2 Three phases of T-cell activation process

T-cell priming and formation of immunological synapse is a very dynamic process involving several successive stages that all together last for a relatively long time, approximately 24-48 hours. Using an *in vivo* model of T-cell activation, Ulrich von Andrian and colleagues (Mempel et al., 2004) distinguished 3 phases occurring during T-cell priming by DCs. Naïve T-cells leave the thymus and migrate into secondary lymphoid organs (lymph nodes and spleen) where they scan the surface of APCs for presence of cognate ligands. In the first phase, T-cells and DCs form short-lived contacts lasting approx. 5-30 minutes. Initial contacts induce an early T-cell activation which is sufficient for expression of early activation markers like CD44 and CD69, but not for upregulation of IL-2α-receptor subunit (CD25). After this initial phase, T-cell motion slows down and stable conjugates between T-cells and DCs are formed. These interactions, characteristic for the phase two of T-cell activation process, continue for about 16 hours, when almost all activation markers on T-cell surface, including CD25, are readily detectable. However, even though the formation of mature immunological synapse with clearly defined borders of Supramolecular Activation Cluster (SMAC) in contact zones is completed (Lee et al., 2002), T-cell proliferation is still not initiated or is on very low levels. The third phase continues approximately one day after naïve T-cell entered lymph nodes and spleen. T-cell:APC conjugates dissociate and fully activated T-cells produce IL-2 cytokine, rapidly divide and leave secondary lymphoid tissues.

2.3 The structure of the immunological synapse.

Interface structure of apposing membranes formed during the initial stages of cell-cell interaction between APC and T-cell is called an immunological synapse (Grakoui et al., 1999). Lymphocyte polarization and co-clustering of important signalling molecules and cytoskeleton compounds into T-cell:APC contact zone occur as a response to recognition of specific antigen-MHC complexes by antigen-specific T-cells. Consistent with cellular events accompanying T-cell priming, several phases recognized on molecular level are distinguished during the formation of IS [reviewed in (Friedl et al., 2005)]. Briefly, during the initial phase, T-cells scan APCs for the presence of cognate antigens. Once they find it, interaction of T-cell adhesion molecules LFA1 and ICAM1 with their ligands expressed on APCs transiently retards the motion of T-cells. Phase 2, which follows, is the most important one and will be reviewed in detail later. Suffice to mention at this point that within a few seconds after T-cell:APC contact is established, multiple signalling pathway are triggered, resulting in the activation of a cascade of protein-protein interactions and cytoskeleton reorganization. Many positive and negative regulatory as well as feedback mechanisms controlling the dynamics, accuracy and amplitude of T-cell activation are in place (Fig 2.1A). Transition into the third phase is visible in about 30 minutes after antigen recognition and is sustained for several hours. At this point, the maturation of IS is already completed (Lee et al., 2002), because T-cell-APC contact site exhibits well-organized circular structures with nearly complete segregation of clustered molecules. Kupfer and colleagues (Monks et al., 1998) modelled the mature IS as a structure consisting of three concentric rings: TCR/CD3 complex, CD28 receptor and PKCθ are localized in a central zone, known as a central supramolecular activation cluster (c-SMAC); LFA-1 adhesion molecules and CD4/Lck complexes are predominantly located in the peripheral ring surrounding c-SMAC (p-SMAC); and a distal zone containing mainly large molecules like CD45 (d-SMAC) [reviewed in (Huppa and Davis, 2003)] (Fig. 2.1B).

The above described distribution of signalling elements into concentric rings of SMAC is in most cases transient as these components can move in or out from one zone to the other. While the nature of these translocations is still obscure, they are required for delivery of regulatory and enzymatic functions of these signalling molecules. The last two phases (phase 4 and 5) contribute to downregulation and breakdown of IS. TCR and LFA1 dissociate from the contact zone and negative regulators, such as CTLA-4, are

upregulated. Concomitantly, motility of T-cells is renewed and their proliferation is initiated.

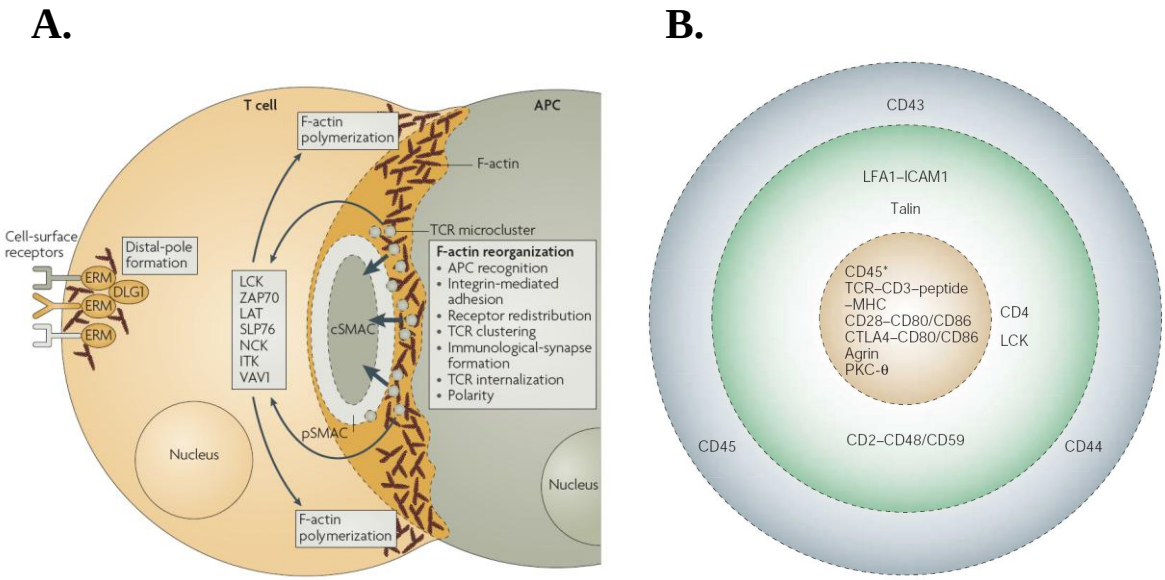


Figure 2.1 Formation and composition of Immunological synapse. **A.** T-cell activation induced F-actin cytoskeletal rearrangement and polarization. The recruitment and activation of T-cell proximal signalling elements like Lck, ZAP70, LAT, SLP-76, Nck, Itk and Vav predicates the formation of immunological synapse which is dependent on F-actin polymerization. F-actin rearrangement is also required for the formation of new TCR microclusters which sustain TCR signalling as well as for the assembly of DPC. This complex, consisting of ERM and DLG1 proteins, functions as a depository area for negative regulators of T-cell signalling (taken from Billadeau et al., 2007). **B.** Crossection of T-cell:APC contact site. Three zones of SMAC are identified – *central (c-SMAC)*: consisting mainly of TCR-MHC, CD28-CD80/CD86, PKCθ [brown]; *periferal*: co-receptors CD4/8, Lck, LFA1-ICAM1 [green] and *distal*: mainly CD45 [blue] (taken from Huppa and Davis, 2003). *DPC-distal pole complex; SMAC- supramolecular activation cluster;*

2.4 Molecular signalling pathways in T-cell activation

Simplified cartoon of T-cell signalling pathways are schematically illustrated in Figure 2.4 (page 20). Three critically important modules control the T-cell activation process: (i) Lck and Fyn regulation module; (ii) Signal triggering module; (iii) Signal diversification and regulation module. Brief description of these modules, characterization of their critical components and mechanism(s) controlling and integrating their effector functions are discussed below.

Of note, even though molecular mechanisms operating during the activation of $CD4^+$ and $CD8^+$ T-cells are analogous, we emphasize that mechanisms of activation and their molecular elements described in following sections are largely derived from studies using $CD4^+$ T-cells, and thus mostly relate to signalling events occurring in these cells.

2.4.1 Lck and Fyn regulation module

As Lck and Fyn provide critical enzymatic activities required for the initiation of proximal TCR signalling, activation and delivery of their functions are tightly regulated. Structure and mode of activation of Lck as the prototype of SFKs as well as regulatory molecules controlling the process of Lck activation are described below.

2.4.1.1 Structure of Lck

Lymphocyte-specific protein tyrosine kinase (Lck) plays an indispensable and crucial role in T-cell maturation and signalling. In its absence, thymocytes development as well as T-cell responses are severely compromised (Molina et al., 1992) what leads to various forms of immunodeficiency and/or autoimmunity diseases (Methi et al., 2005; Methi et al., 2007).

Molecular weight of Lck is approximately 56 kDa and its structure, as illustrated in Figure 2.2, is highly conserved among other SFK members (Boggon and Eck, 2004). Lck has a short N-terminal region posttranslationally modified by double acylatation. These lipid modifications not only predispose Lck to the inner membrane leaflet of plasma membrane but also provide a targeting signal for docking it into distinct membrane micro-compartments called lipid rafts. The unique domain at N-terminal end of Lck is the least homologous among SFKs and facilitates a non-covalent interaction with co-receptors CD4 and CD8. The unique domain is followed by Src homology 3 (SH3) and SH2 domains involved in mediating intramolecular and intermolecular interactions via binding to proline rich sequences and phosphotyrosine motifs, respectively. Following is the kinase domain and the C-terminal tail important for catalytic activity of Lck and its negative regulation, respectively.

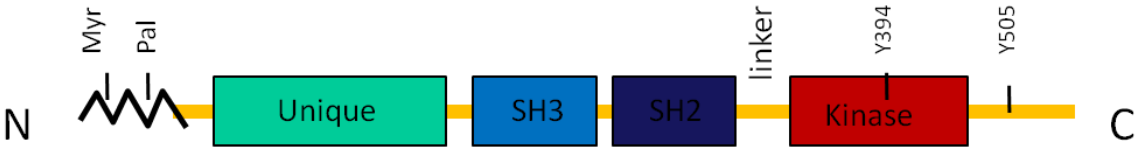


Figure 2.2 Structure of Lck kinase. Lck contains several regions and domains which are highly conserved among members of Src family tyrosine kinases: a short N-terminal, lipid-modified region with one site for myristoylation (G2) and two potential sites for palmitoylation (3C and 5C), a unique domain containing the binding site for co-receptor molecules CD4 and CD8, Src homology 3 (SH3) and SH2 domains recognizing prolin-rich and phosphotyrosine motifs, respectively, a linker region, a kinase domain responsible for catalytic function, and a C-terminal tail involved in negative regulation of function via tyrosine at position Y505. The activatory tyrosine is positioned within the kinase domain at position 394 (Y394).

2.4.1.2 Mechanism regulating kinase activity of Lck

Intrinsic catalytic activity of kinase domain of Lck is negatively controlled via formation of two weak intramolecular interactions: (i) between the C-terminal pY505 and SH2 domain and (ii) SH3 domain and polyproline-rich linker region. In this scenario, the kinase domain is held in an inactive, repressed conformation and the substrate accessibility is blocked (Mayer, 1997). While the phosphorylation of Y505 residue is under the control of C-terminal Src kinase (Csk), pY505 dephosphorylation is executed by CD45 phosphatase which counteracts the action of Csk and results in open "primed" conformation of Lck (Fig. 2.3) (Hermiston et al., 2002; Palacios and Weiss, 2004). The latter, coupled with the transphosphorylation of positive regulatory tyrosine residue Y394 is required for full kinase activity (Veillette et al., 2002).

In summary, CD45 acts as positive regulator of Lck activity in contrast to Csk playing a negative regulatory role. How exactly is the equilibrium between these two opposing regulations established and how is this dynamic process controlled is not well understood. In this context it has been suggested that other proteins like the adaptor scaffold protein PAG/Cbp, PEP phosphatase, adaptor proteins TSAD and Unc-119, to mention just a few, are also involved in Lck and Fyn regulatory loops (Brdicka et al., 2000; Filby et al., 2007; Kawabuchi et al., 2000) (Fig. 2.3 and 2.4A). It is important to mention at this point that membrane microdomains, lipid rafts, add an additional level of complexity to these regulations as they serve as a platform for regulation of spatial

distribution and TCR-induced clustering and translocation of these effector molecules. The role of LR in proximal TCR signalling is highlighted below, in the section 2.7.2.

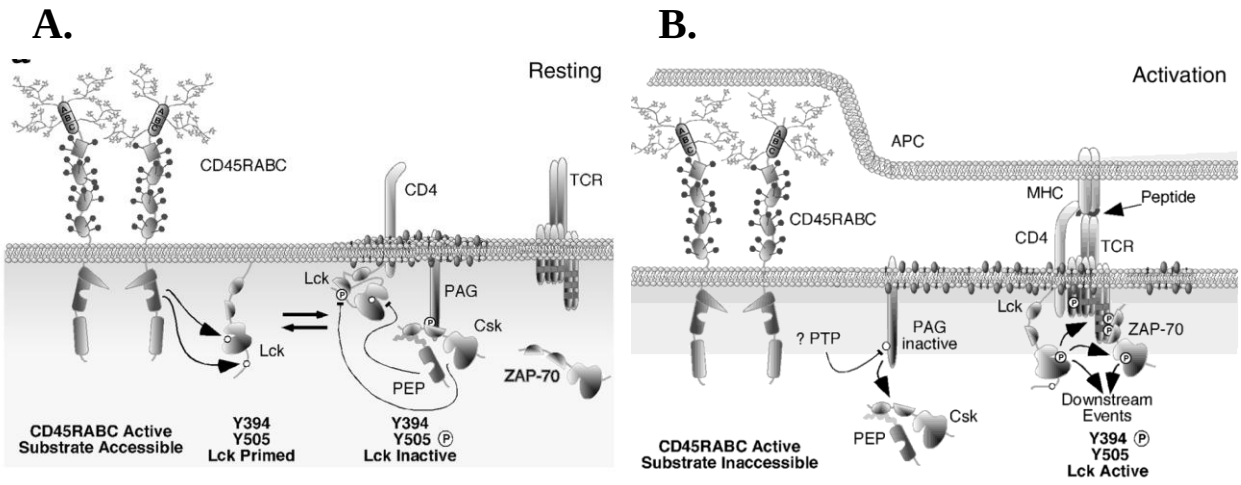


Figure 2.3 Modelling the regulation of Lck activity: Lck has two important regulatory tyrosines – at position 505 (Y505, negative regulatory site) and at position 394 (Y394, positive regulatory site) phosphorylation of which regulate the activation status of Lck, negatively and positively, respectively. **A.** In resting T-cells, Lck Y505 tyrosine is phosphorylated by Csk kinase and pY505 then binds on self SH2 domain, keeping the kinase structure in *"closed"*, autoinhibited, inactive conformation. Dephosphorylation of Y505 tyrosine by CD45 phosphatase *"primes"* Lck but the kinase is not fully active as the activatory tyrosine Y394 is not phophorylated. **B.** Upon TCR engagement and T-cell activation, primed Lck is clustered around TCR, what leads to its autotransphosphorylation on the activatory Y394 and this modification is required for the fully "*active"* state of Lck. In addition, upon activation, CD45 or some other phosphatase(s) can dephosphorylate the adaptor protein PAG, resulting in the displacement of Csk from PAG and thus from the vicinity of Lck, keeping Lck active and disallowing its downregulation. (Taken from Hermiston et al., 2002).

Besides Lck, another SFK member - Fyn is important for T-cell signalling and activation. It was previously believed that its function is more redundant and overlapping with that of Lck, however data accumulated in past few years indicate that it can play a distinct and more specific role(s) in proximal signalling, for example interaction with cytoskeleton regulators (Badour et al., 2004). In addition, it has been demonstrated that activation of Lck and Fyn kinases is sequential and unidirectional, Lck first then Fyn, and is temporarily and spatially uncoupled (Filipp and Julius, 2004) (see section 2.8). Moreover, many Lck- and Fyn-specific interaction partners were identified what underpins their independent and essential roles in T-cell activation (Zamoyska et al., 2003).

2.4.2 Signal triggering module

An early T-cell activation process is control through several negative and positive regulatory feedbacks. It can arbitrary be divided into two phases: (i) proximal early activation signals occurring during the first few 3-5 minute and late 5-30 minutes period, when the mature IS forms and production of IL-2 cytokine is induced. Brief description of TCR, auxiliary molecules and the mechanism underpinning the generation of TCR emanating signals is provided below (Fig. 2.4).

2.4.2.1 T-cell receptor complex

T-cell receptor (TCR) together with CD3 chains form TCR/CD3 complex which is responsible for antigen recognition and transmission of this signal to downstream effectors. Recognition ability is provided by TCR/MHC interaction. TCR is heterodimeric molecule where both α and β chains consist of variable and constant regions. Somatic rearrangement of TCR occurs during thymocyte development in the thymus and enables to generate an enormous repertoire of TCR variants able to recognize practically any antigenic peptide expressed in the context of MHC on APCs. As α and β chains of TCR have no functional connection to intracellular space, an engagement of TCR is translated to intracellular signalling elements via CD3 chains γ, δ, ε and ξ. All of them contain tyrosine phosphorylation sites within Immunoreceptor Tyrosine-based Activation Motifs (ITAM), which serve as docking sites for ξ-chain associated protein of 70kDa (ZAP-70) kinase.

2.4.2.2 Auxiliary signalling elements

Many other proteins contribute to early steps of T-cell signalling. CD4 and CD8 co-receptors are able to bind through their extracellular portions to MHCII and MHCI, respectively, and significantly enhance T-cell responses to presented antigen via delivery of Lck to vicinity of TCR/CD3 complex. Phosphatase CD45 plays an important part in positive regulation of Lck activity by dephosphorylating its C-terminal negative regulatory tyrosine Y505. Integrin LFA-1 provides adhesive function through binding to ICAM ligand expressed on APCs and thus mediating T-cell:APC interaction. Last but

not least are co-stimulatory molecules CD28 and CTLA-4 involved in the regulation of T-cell activation.

2.4.2.3 Proximal TCR signalling

First seconds after TCR/MHC engagement are crucial for development of downstream signal response and the full activation of T-cells (Fig. 2.4B). The most proximal, biochemically detectable cellular event occurring after antigen recognition is the induction of tyrosine phosphorylation by two SFKs, Lck and Fyn. Upon activation, extracellular portion of CD4, the transmembrane co-receptor associated with Lck intracellularly, binds simultaneously with TCR to pMHCII complex and juxtaposes Lck to the proximity of TCR/CD3 chains. Both kinases contribute to phosphorylation of tyrosine residues on ITAMs within the chains of TCR/CD3 complex. Once phosphorylated, these motifs serve as docking sites for SH2 domain of ZAP-70 (member of Syk family kinases). After recruitment to ITAM motifs, Lck further phosphorylates and thus activates ZAP-70 as well as other Protein Tyrosine Kinases (PTKs) of Tec family. The phosphorylation level in T-cell is rapidly increasing and culminates in activation of other downstream signalling proteins and pathways.

2.4.3 Signal diversification and regulation module

After their activation, ZAP 70 and Lck phosphorylate several target proteins resulting in diversification of regulatory signalling pathways (Fig. 2.4C). Linker for activated T-cell (LAT) is the primary target for ZAP-70 phosphorylation. It serves as the scaffold protein for recruiting additional proteins, including phospholipase C gamma (PLCγ), Grb-2, Gads, Cbl and PI3K via their SH2 and SH3 domains. This multimeric protein complex is the initiation point for activation of several signalling pathways. Critical in this sense is the recruitment and activation of PLCγ , Ras and Vav signalling pathways resulting in the activation of NF-AT, NF-κB and AP-1 transcription factors which then cooperatively bind to IL-2 promoter and synergistically induce the transcription and production IL-2 cytokine which is required for T-cell proliferation (Monks et al., 1997; Nel, 2002). LAT recruitment of GADS-associated multiproten complex consisting of SLP-76, VAV, ITK, NCK, WASP and PAK is responsible for initiation of actin

remodelling and cytoskeleton rearrangement (Billadeau et al., 2007). PI3K attachment to LAT activates AKT kinase, which promotes cell survival via upregulation of anti-apoptotic proteins BAD and BCL-xL. Thus, TCR activation leads to a multifaceted cellular response leading to global changes in gene expression and ultimately to cytoskeleton reorganization, proliferation and differentiation of T-cells.

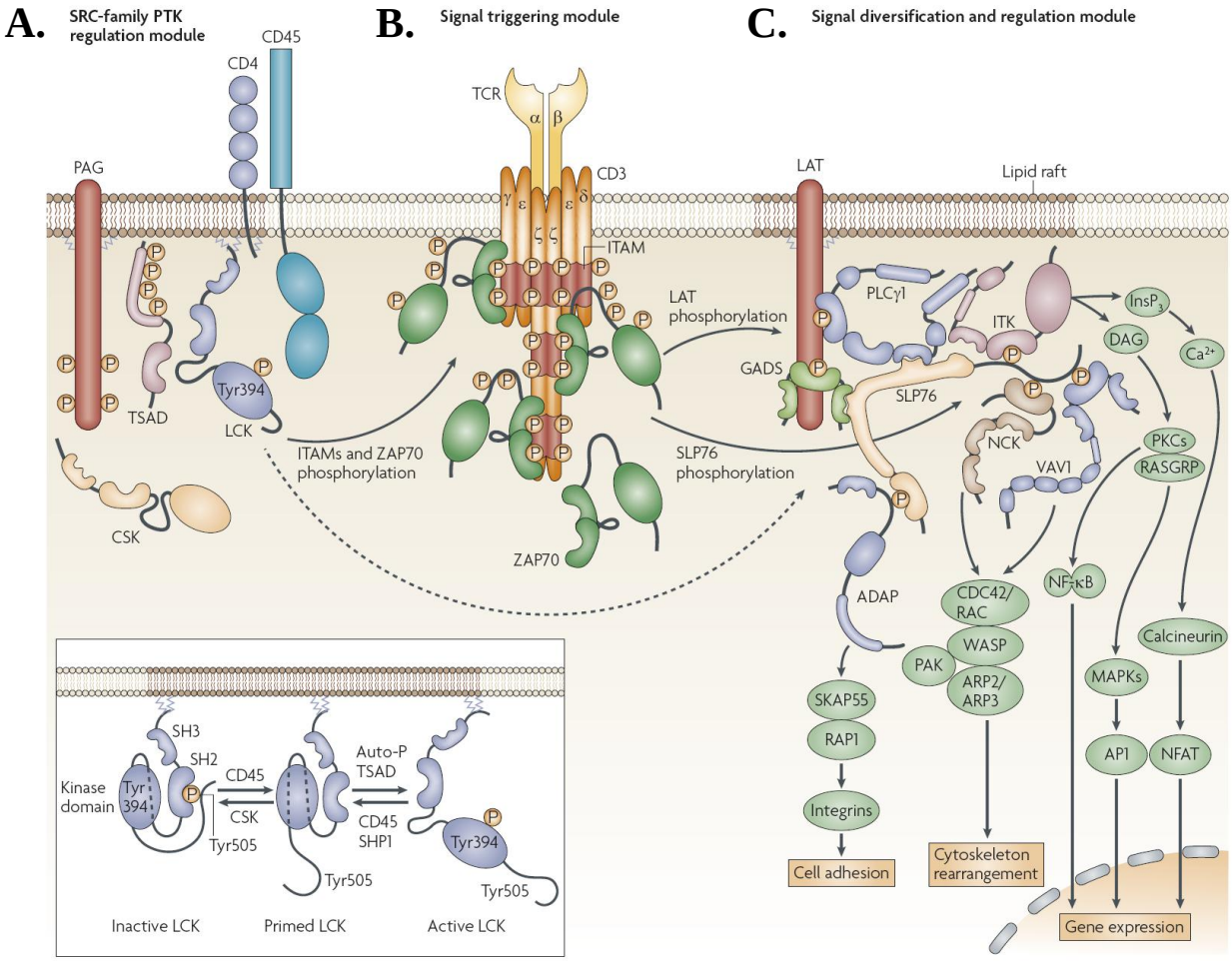


Figure 2.4 T-cell activation signalling pathways. Initiation of T-cell signalling could be divided into following three modules. **A.** The first module contains regulatory units controlling Lck activation. Inactive Lck in resting T-cells is in "*closed*" conformation which retains the phophorylation of negative regulatory tyrosine (Y505) at Lck C-terminal tail mediated by Csk. Phosphatase CD45 opposes Csk function by dephosphorylating pY505, thus enabling the opening of Lck structure into "*primed*" conformation. **B.** The Second module features clustering of Lck in the proximity of TCR/CD3 complexes upon TCR engagement. Due to induced clustering, Lck molecules transphophorylate each other on their activatory tyrosine (Y394) and achieve fully "*active*" state. Kinase active Lck then phosphorylate ITAM motifs of CD3 chains which serve as docking sites for ZAP-70 kinase which is then recruited and further activated by Lck phosphorylation. **C.** The schematics of the third module depicts the phosphorylation of adaptor proteins LAT and SLP-76 by active ZAP-70 and Lck resulting in diversification of several regulatory signalling pathways responsible for initiating changes in gene expression, cytoskeleton rearrangement and cell adhesion. (Taken from Acuto et al., 2008).

2.4.4 CD28 – important co-receptor molecule

CD28 plays an important role in T-cell activation as it provides the secondary co-stimulatory signal upon its interaction with members of B7 family receptors expressed on APC. In the absence of this signal, TCR/CD3 engagement results in T-cell anergy or even death. If both TCR- and CD28-mediated signals are provided, IL-2 production is significantly increased.

It has been shown that CD28 proline-rich sequences can directly interact with SH3 domain of Lck and Fyn. Binding of Lck to CD28 seems to be a necessary prerequisite for sustained Lck phosphorylation of activatory Y394 in the catalytic domain (Holdorf et al., 2002). In addition to Lck, CD28 binds also to Itk and PI3K, but a disruption of these interactions did not provide any obvious signalling phenotype (Nel, 2002). It is assumed that several, yet unknown compensatory mechanisms and cross-talks operate between TCR and CD28 triggered pathways.

Recruitment of CD28 and its localization in cSMAC requires sustained TCR signalling and interaction with B7 ligands on APC (Sanchez-Lockhart et al., 2008). Subsequently, CD28 induces translocation of PKCθ to the same sub-region of IS what results in activation of NF-κB transcription factor (Huang et al., 2002; Yokosuka et al., 2008). The mechanism responsible for translocation of CD28 into IS depends on cytoskeleton reorganization and local accumulation of lipid rafts. Molecular machinery involved is veiled but one protein candidate, filamin A (FLNA), was recently discovered. FLNA is a cytoplasmic protein able to bind to both the cortical actin and CD28. Downregulation of FLNA in Jurkat T-cells results in abrogated CD28 localization into IS (Tavano et al., 2006).

2.5 Role of cytoskeleton in T-cell activation

The proper functioning of cytoskeleton is absolutely essential for T-cell activation. Cellular cytoskeleton consists of three distinct systems: actin, microtubules and intermediate filaments. Together they regulates critical physiological processes including T-cell movement and cellular polarization during IS formation through integrin-mediated adhesion and signal sequestration (Billadeau et al., 2007; Burkhardt et al., 2008).

So far, it seems that the most important contribution comes from filamentous actin (F-actin). Its explosive polymerization upon TCR engagement is responsible for T-cell polarization, the formation of IS and the establishment of Distal Pole Complex (DPC). In addition it supports the sequestration and/or translocation of signalling molecules into their interaction sites upon T-cell activation. Signalling cascade Lck-ZAP70-SLP-76-Vav is required for this process. Ultimately, Vav activates small RHO-GTPase CDC42 and RAC1 and they, via intermediaries, activate actin regulatory protein complex ARP2/3 which directly regulates actin polymerization. F-actin is also involved in the spatial re-localization of negative regulators during the onset of T-cell signalling into DPC. Actin linker proteins ezrin and moesin, which belong to ERM family, binds DPC-associated proteins CD43 and EBP50 and they, in turn, interact with SFK negative regulator Phosphoprotein Associated with GEMs (PAG) (Brdickova et al., 2001). Moreover, as Ezrin transiently translocates to IS during the early phase of T-cell activation, it suggests, that it might be involved in the delivery and/or removal of a cargo to or from IS, respectively (Shaffer et al., 2009). Besides actin polymerization, T-cell activation is accompanied by the reorientation and translocation of Microtubule-Organizing Center (MTOC) to the proximity of T-cell:APC contact zone. Reorientation of MTOC towards IS seems to be important as microtubules function as a tubular network supporting a directed secretion of transportation vehicles containing various lymphokines (IL-2, IL-4) or cytotoxins (perforins, granzymes) towards APC surface or target T-cell (Stinchcombe et al., 2006).

2.6 Negative regulators of T-cell activation

To downregulate signalling pathways controlling T-cell activation, several inhibitory feedback mechanisms operate to maintain an equilibrium and T-cell homeostasis (Acuto et al., 2008). As a good example of negative feedback mechanism serves the phosphorylation of PAG by kinase active Fyn which in turns leads to the recruitment of Csk, the main negative regulator of Fyn and Lck. (Filby et al., 2007). Furthermore, the surface expression of negative T-cell regulator CTLA-4 competes with CD28 co-stimulatory receptor for B7 ligands. CTLA-4 exerts the negative regulatory function via its association with phosphatase SHP-2 which binds to ξ-chains of TCR and dephosphorylates them. A negative function has also been ascribed to protein Cbl, the ubiquitine E3 ligase, responsible for enhanced degradation of PTKs. Tyrosine

phosphatase SHP-1 participates in downregulation of T-cell activation through binding to receptors containing Immunnoreceptor Tyrosine-based Inhibition Motifs (ITIMs) and by dephosphorylating activatory pY394 of Lck. Many other proteins involved in downregulation of T-cell activation have been recently cloned and characterized, for example DOK, HPK1 and STS to list just a few (Acuto et al., 2008).

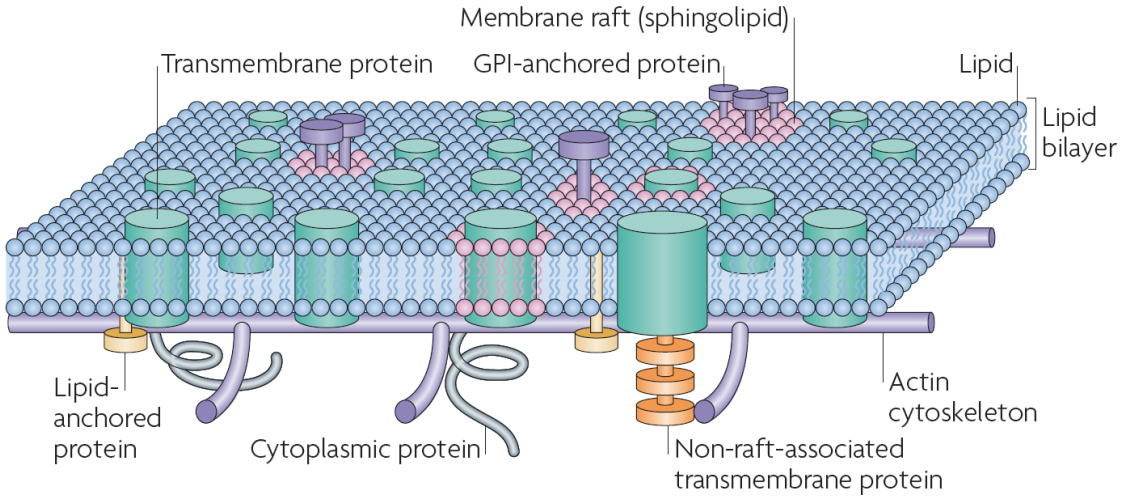


Figure 2.5 The plasma membrane. The plasma membrane has an asymmetric composition of lipids in outer (sfingolipids, glycosphingolipids, phosphatidyl-choline) and inner (glycerophopholilids) leaflets. Proteins and lipids non-covalently associate and can form membrane rafts (pink). Rafts are very dynamic, sterol- and sphingolipid-enriched islands immobilizing glycosyl-phosphatidylinositol (GPI)-anchored proteins (purple). Subcortical actin and actin-binding proteins play the key role in regulation of membrane-raft dynamics interacting with lipids of inner leaflet (Taken form Viola and Gupta, 2007).

2.7 Lipid rafts

2.7.1 General characterisation of lipid rafts

In 1972 Singer and Nicolson presented the "fluid mosaic" model of plasma membrane where phospholipids, basic building stones of membrane, exhibit a very rapid lateral movement along the membrane bi-layer (Singer and Nicolson, 1972). According to this model, lipids are distributed randomly and form liquid disordered structures (Ld). However, a new plasma membrane model (Fig. 2.5), acknowledging the existence of very small, heterogeneous clusters consisting of sphingolipids with long saturated acyl chains and sterols has been proposed (Simons and Ikonen, 1997). These membrane micro-heterogeneities, forming liquid-ordered structures (Lo) are called lipid rafts (LR)

or Detergent Resistant membrane Microdomains (DRMs). "Lipid raft hypothesis" defines LR as lipid clusters insoluble in mild non-ionic detergents at low temperature (for example Triton X-100 or Brij-58 at 4°C) (Simons and Toomre, 2000). Even though this definition is operational (definition of LR is based on a procedure used for their isolation) (Methods section 3.12), there is a general consensus among "raftologist" (scientists studying LR) that LR concentrate and co-localize proteins and protein complexes involved in the process of T-cell activation. Despite the fact that in the last few years existence of LR has been quite well documented (Viola and Gupta, 2007), their functional and physiological importance still awaits its full justification (Munro, 2003; Shaw, 2006). Important questions concerning their formation, lifetime, size, dynamics, composition, turnover, movement and importance remain to be answered.

2.7.2 LR in T-cell activation

Recently published studies suggest that LR regulate spatial segregation of signalling molecules on the plasma membrane before and shortly after T-cell activation (Manes and Viola, 2006; Rodgers et al., 2005; Viola and Gupta, 2007). During lymphocytes migration from blood stream to their target destination, T-cells exhibit morphological and functional asymmetry changes called polarization. This process involves a reorganization of subcortical actin cytoskeleton and a polarized assembly of LR. The formation of two cellular poles – leading edge at the cell front and uropod at the rear, correlates with distinct distribution of LR subtypes: U-rafts at the uropod, characterized as GM1-containing rafts and GM3-containing L-rafts at the leading edge. Differences in composition of L- and U-rafts could signify their role in the recruitment and segregation of two sets of proteins exhibiting specific and mutually incompatible functions. Usually proteins involved in actin reorganization are targeted to the leading edge and include chemotactic receptors and CD28. Receptors and proteins playing a role in cell adhesion (ICAM1-3, CD43, CD44, ERM) segregate preferentially to the uropod.

During formation of IS, small LR microclusters on both sides of polarized cell coalesce into one larger macrodomain, thus targeting and concentrating signalling proteins to IS or to DPC (Rodgers et al., 2005). The mechanisms underpinning regulated compartmentalization of signalling elements are not known, nevertheless available data strongly suggest that raft dynamics depends mainly on F-actin rearrangements. It is

supported by interactions of actin with several LR-residing molecules thus mediating actin attachment to LR. For example, as mentioned previously, FLNA cross-links actin filaments to generate and sustain sub-cortical actin cytoskeleton and it simultaneously interacts with the co-stimulatory molecule CD28 localized in LR (Tavano et al., 2006). Another example is CD28 engagement-induced activation of Vav-dependent actin polymerization pathway. Furthermore, ERM proteins are also involved in interaction with actin and signalling molecules associated with LR like PAG (Brdickova et al., 2001). All together, these data point to the essential importance of LR and cytoskeleton cooperation in regulation of T-cell migration and activation.

Propensity of proteins to distribute to LR is largely dictated by the structure and type of their transmembrane domain and/or posttranscriptional modifications. As an example of relevant LR-targeting modifications are SFKs Lck and Fyn (double acylation of N-terminal segment) and a cysteine palmitoylation of adaptors LAT and PAG. By the same token, GPI anchor also target proteins to LR due to its resemblance to sphingolipids. It is assumed that an enrichment of other proteins, like TCR and B-cell receptor, in LR is induced upon activaton of T-cells by virtue of their complexation or clustering with LR-targeted proteins.

Recently a new model of proximal T-cell activation demonstrating an involvement of LR in this process has been proposed (Filipp et al., 2004; Filipp et al., 2003) and is described in following section.

2.8 Lck-dependant Fyn activation model

Lck-dependent Fyn activation model, describing non-redundant and interdependent functions of two Src family tyrosine kinases Lck and Fyn that predicate the generation of the most proximal signals emanating from the antigen receptor complex in T-cells has been described (Fig. 2.6). In this model, LR not only function to segregate Lck and Fyn but also couple the process of membrane translocation with temporal and spatial regulation of their enzymatic activities. Specifically, in primary resting T-cells, 75-95% of Lck resides outside LR and is physically segregated from more than 98% of Fyn residing in LR. Co-aggregation of TCR and CD4 results in Lck activation within seconds outside LR, followed by its translocation into LR and the activation of co-localized Fyn. The kinase activity of Fyn was observed to peak at 30 to 90 seconds

after TCR/CD4 co-aggregation (Filipp et al., 2003). These results define Lck as a mobile signalling element and underscore the importance of targeting its kinase activity in delivery of function (Filipp et al., 2004; Filipp et al., 2003).

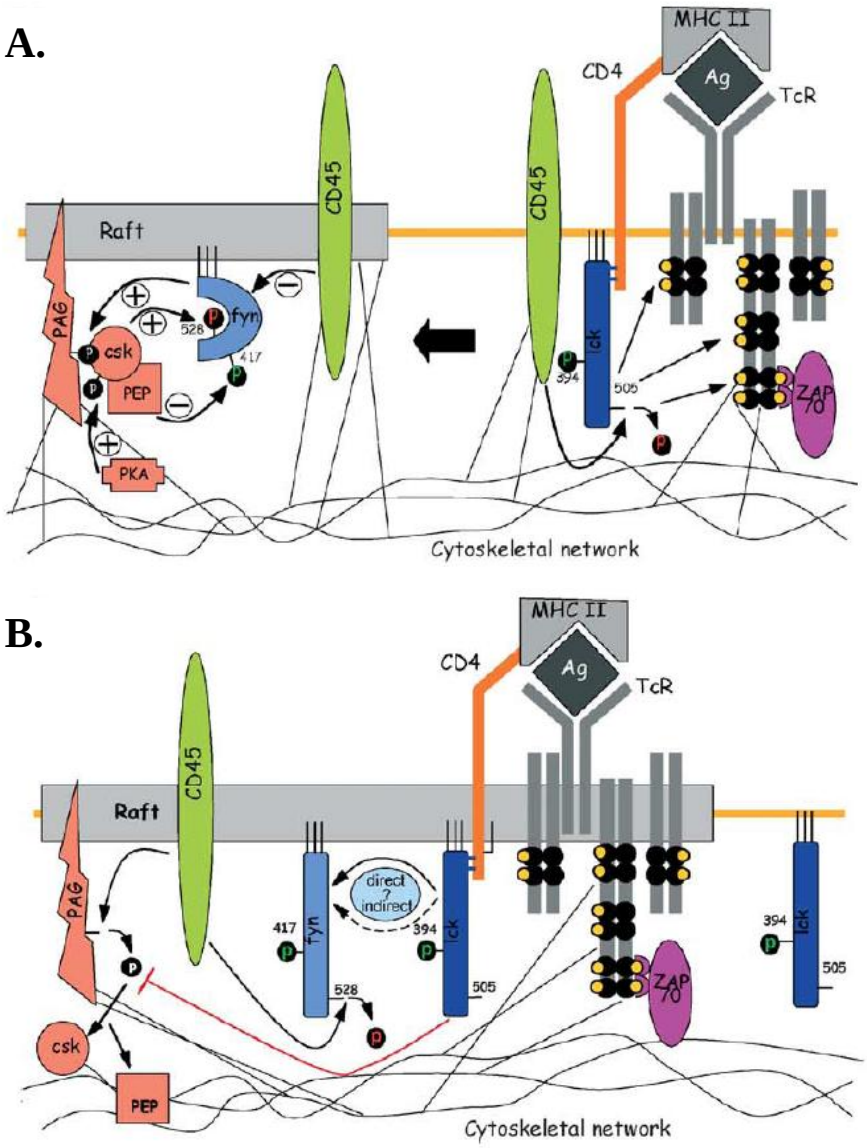


Figure 2.6 Model of Lck dependent Fyn activation through translocation of Lck kinase into LR. This model is based on the fact that in resting naive $CD4^+$ T-cells 75-95% of Lck resides outside of LR whereas more than 98% of Fyn is in LR (Filipp et al., 2003) **A.** Upon TCR engagement, dephosphorylation of pY505 by CD45 phosphatase and transphosphorylation of Y394 activates Lck. Fully active Lck initiates ITAM phosphorylation and recruitment of ZAP-70 kinase to CD3 complexes. At same time, Fyn is localized in LR, where its activity is negatively regulated by PAG/Csk/PEP feedback mechanism which is at least partly controlled by PKA. **B**. During the first 90 seconds after T-cell activation the entire TCR/CD3/Lck complex somehow translocates into LR, where it inhibits the function of PAG/Csk/PEP complex and subsequently activates Fyn. While the mechanism of translocation remains largely unknown, an involvement of cytoskeletal network is expected. (Taken from Filipp and Julius, 2004).

The subsequent structure-function analysis towards characterizing the mechanism supporting Lck partitioning to LR and its capacity to activate co-localized Fyn showed that only LR-associated, kinase active Y505F-Lck reciprocally co-immunoprecipitates with and activates Fyn (Filipp et al., 2008). Mutational analyses revealed a profound reduction in the formation of Lck-Fyn complexes, and Fyn activation, using kinase domain mutants K273R and Y394F of Y505F-Lck, both of which have profoundly compromised kinase activity. The only kinase active Lck mutants tested that revealed impaired physical and enzymatic engagement with Fyn were those involving truncation of the C-terminal sequence YQPQP. Remarkably, sequential truncation of YQPQP resulted in an increasing reduction of kinase active Lck partitioning to LR, in both fibroblasts and T-cells. This in turn correlated with an ablation of the capacity of these truncates to enhance TCR mediated IL-2 production. Thus, Lck-dependent Fyn activation is predicated by proximity-mediated transphosphorylation of the Fyn kinase domain; and targeting kinase active Lck to LR is dependent on the c-terminal sequence QPQP (Filipp et al., 2008).

2.9 Identification of candidate proteins involved in translocation of Lck to LR

One prediction derived from the model of Lck-dependent Fyn activation is that the critical regulatory step in activation of Fyn kinase is the translocation of kinase active Lck to LR. However, there is a paucity of information in regard to the mechanism and the molecular machinery underpinning this process.

As described in the previous section, the truncation of the last five C-terminal residues of kinase active Lck (ΔFQPQP) profoundly reduced its partitioning to LR. This suggests that the C-terminal tail of Lck functions as LR targeting sequence. Our working hypothesis is that the FQPQP sequence targets Lck to protein(s) of the cytoskeletal network and supports their phosphorylation. This, in turn, initiates an early and very rapid cytoskeletal network reorganization culminating in the re-distribution of Lck to LR. Consistent with this hypothesis is the inability of constitutively active ΔFQPQP-Lck to phosphorylate several low molecular weight proteins detected in the presence of full length constitutive active Y505F-Lck, thus identifying these phosphoproteins as candidates in targeting Lck to LR (Fig. 2.7A). Comparative 2-dimensional (2D) gel analyses followed by immunoblotting with phosphotyrosine specific mAb 4G10

identified several differentially phosphorylated protein spots (Fig. 2.7B). Some of them have been isolated and are currently being analysed. Closer characterization of one of them, identified as Receptor for Activated C Kinase 1 (RACK1) is the subject of this investigation.

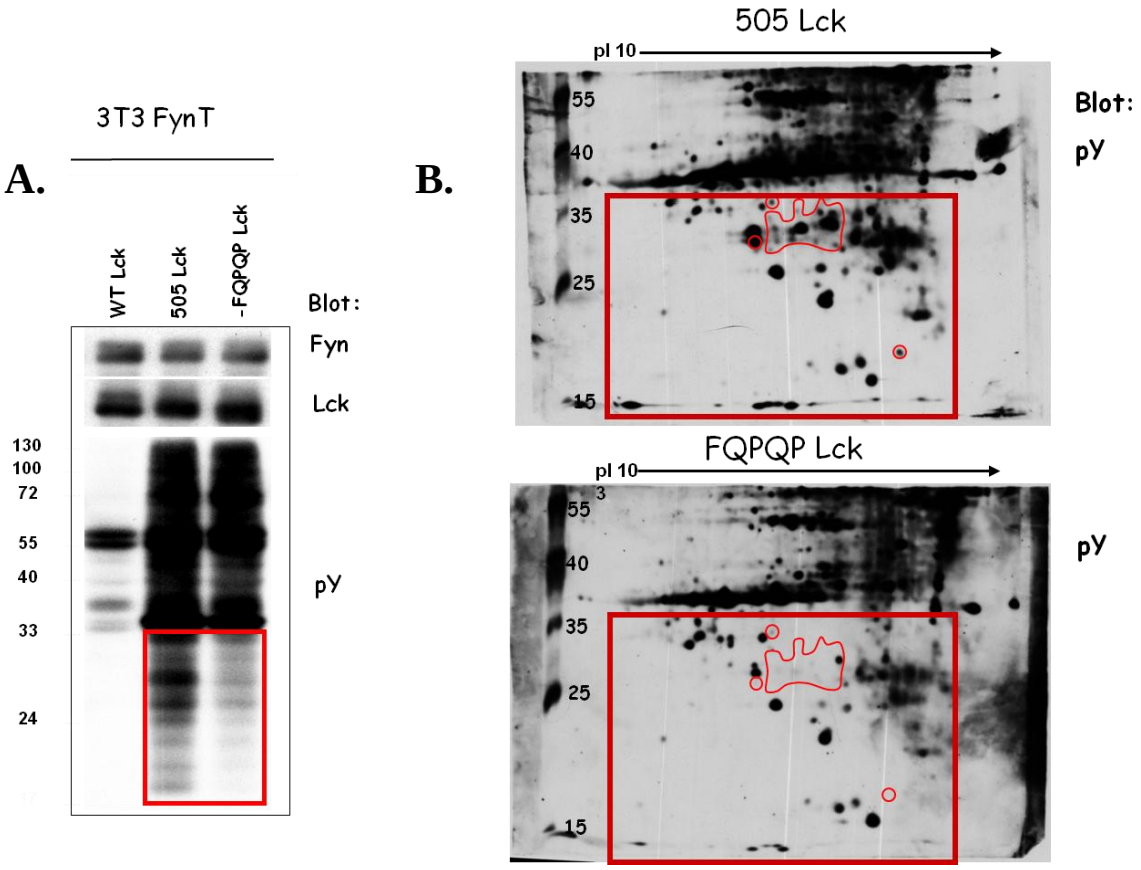


Figure 2.7 Identification of proteins involved in the translocation of Lck kinase into LR. A. The C-terminal sequence (YQPQP) functions as a lipid raft targeting sequence of Lck. Comparative analysis of tyrosine-phosphorylated cellular proteins derived from lysates of 3T3 cells expressing either the constitutive active Y505F-Lck (505 Lck) or the constitutive active ΔFQPQP C-terminal truncate Lck (FQPQP Lck), revealed a failure of FQPQP-Lck to phosphorylate several, mostly low-molecular weight proteins (red rectangle). **B.** To increase the resolution of proteins of interest shown in A. (red rectangle), a comparative 2D-gel electrophoresis was implied. Several spots displaying significant differences in phosphotyrosine content (demarcated by red lines) were isolated, subjected to MS-MALDI analysis and subsequently identified (Filipp et al., unpublished data). Gels shown in A. and B. were blotted with anti-phosphotyrosine-specific antibody (4G10).

2.10 RACK1 – Receptor for activated C kinase 1

A group of functionally homologous proteins, named RACKs, able to bind activated PKCs were first characterized in 1991 (Mochly-Rosen et al., 1991). Tree years later, RACK1 was cloned from rat brain cDNA expression library and characterized as homolog of the β-subunit of trimeric G proteins and also as interaction partner for PKCβII isoform (Ron et al., 1994). Then, additional two RACK-related proteins were identified: RACK2, which binds PKCε (Csukai et al., 1997) and PRKCBP1, the binding partner for PKCβI (Fossey et al., 2000). RACKs do not posses any catalytic activity and are believed to function as the scaffolding and/or adaptor proteins involved in various signalling processes. RACK1 and RACK2 are members of a very ancient protein superfamily, called WD proteins, which consist of several "WD40 repeats", sequences ending with Try-Asp dipeptide (Neer et al., 1994).

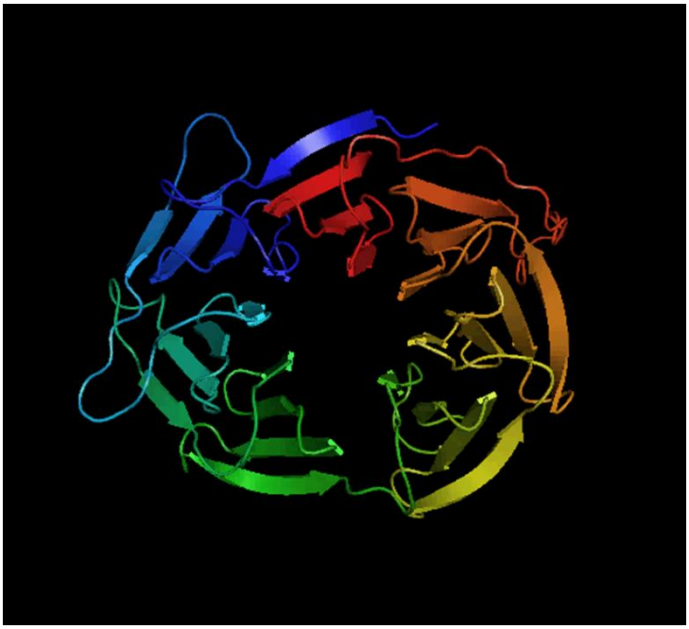


Figure 2.8 The protein structure of RACK1. RACK1 contains seven WD40 domains and each unit consists of three β-sheets and two α-turns. Based on crystallographic data of homologous WD40 motif containing proteins, the tertiary structure of RACK1 protein was deduced using the software program PyMol (Sondek et al., 1996; Wall et al., 1995) and (http://pymol.org/). RACK1 forms seven-blade propeller-like structure where each WD40 domain corresponds to one blade.

RACK1 is 36k Da protein, containing seven WD40 repeats (Fig. 2.8) (Steele et al., 2001). It is evolutionary highly conserved in a wide range of eukaryotes including

plants, fungi and yeast, suggesting that biological function of RACK1 was well enrooted before evolutionary split into plant and animal kingdoms (Neer et al., 1994). RACK1 is express in various tissues in mammals, suggesting its important physiological role in the most cell types. In addition, WD40 domains with the propeller structure, provides RACK1 with multiple binding sites allowing formation of distinct protein-protein complexes what in turns predispose RACK1 to be involved in a broad range of cellular processes.

2.10.1 RACK1 binding partners

Activated Protein Kinase C (PKC) was characterized as the first binding partner of RACK1 (Mochly-Rosen et al., 1991). Notably, as only the activated form of PKC was able to interact with RACK1, it was suggested that RACK1 could serve as a transportation and delivery vehicle for targeting its activated binding partners to the specific intracellular compartments where they execute their effector function (Ron et al., 1994).

This is certainly the case for PKCβII, a serine/threonine kinase, whose activity depends on phospholipids, calcium and mainly on DAG. Upon activation by phorbol esters (e.g. phorbol 12-myristate 13-acetate - PMA) or hormones, an elevated concentration of DAG induces RACK1-PKCβII co-localization which predicates their coordinated translocation and redistribution to a distinct subcellular compartment (Ron et al., 1999) (Figure 2.9A). While mechanisms regulating these dynamic processes are still obscure, multiple protein binding capacity of RACK1's seven WD40 domains most likely contribute to this processes. In this context, several interacting partners, including integrins (Liliental and Chang, 1998), pleckstrin homology (PH) domain-containing spectrin and dynamin-1 (Rodriguez et al., 1999), the cytoskeletal linker protein plectins (Osmanagic-Myers and Wiche, 2004) as well as other proteins (McCahill et al., 2002; Schechtman and Mochly-Rosen, 2001; Sklan et al., 2006) have been characterized and the mechanistic model of their potential contribution suggested.

For example, proteins which PH domain binds to membrane phospholipids are also substrates for PKCβII (pleckstrin, dynamin-1). Thus, RACK1, associated with kinase active PKCβII and anchored by these proteins to the plasma membrane functions as an

adaptor protein allowing its interacting kinase to phopshorylate its substrates and activate them. Similarly, β-integrins which play an important role in cell adhesion, and are involved in cytoskeleton rearrangement can also be phosphorylated by activated PKC. Thus, RACK1 may also regulate cell adhesion by linking PKC and β-integrins together (Cox et al., 2003; Liliental and Chang, 1998). Consistent with this data is the role of RACK1 in activation of adhesion-induced MAPK/ERK pathway. In this scenario RACK1 acts as a scaffold protein localizing MAPK/ERK cascade to focal adhesion sites (Vomastek et al., 2007).

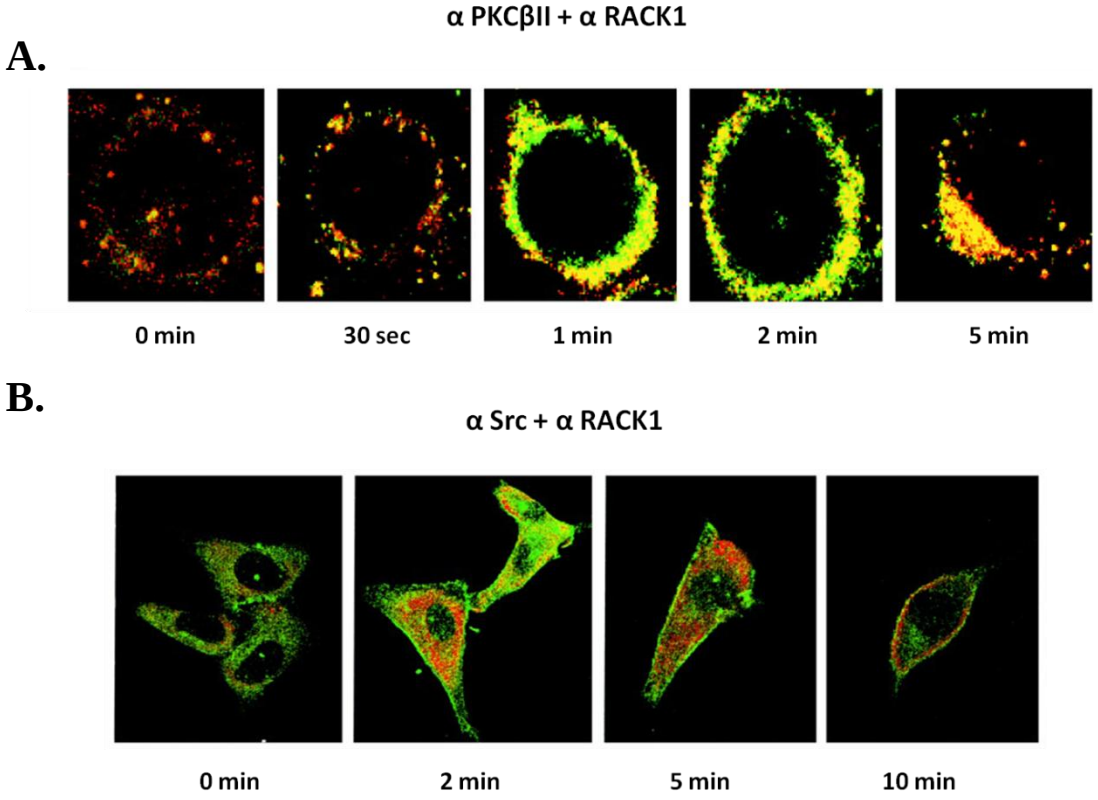


Figure 2.9 Time dependent co-localization and movement of RACK1 with PKCβII and Src upon cell treatment with NPA and PMA respectively. A. CHO/D2L cells were treated with trihydroxy-N-propyl-noraporphine hydrobromid (NPA) and probed with anti-PKCβII (red) and anti-RACK1 (green). The activation induces co-localization of both proteins (see 1 and 2 min. time points) prior to their co-redistribution into a restricted subcellular compartment (5 min time point) (Taken from Ron et al., 1999). **B.** NIH3T3 cells overexpressing Src kinase were treated with PMA for the time indicated and immunostained for Src (green) and RACK1 (red). 10 minutes after PMA treatment Src and RACK1 were found translocated to the plasma membrane where they co-localized. (Taken from Changet al., 2001).

Plectin-mediated interaction of RACK1 with cytoskeleton was also reported (Osmanagic-Myers and Wiche, 2004). Plectin is a cytoskeletal linker protein able to dynamically interact with all three major cytoskeletal elements (actin, microtubules and intermediate filaments) and with components of cell adhesion machinery (Svitkina et al., 1996; Wiche, 1998). Importantly, RACK1-plectin-cytoskeleton interactions were observed only after PKC activation-induced translocation of RACK1 to plasma membrane, where co-localization of these proteins was detected.

Importantly, as WD40 domains can form hetorodimers, potential number of interacting proteins containing this domain can be quite large. For example as previously described, RACK1 is the homolog of β-subunit of heterotrimeric G proteins. G proteins trigger various extracellular signal-induced signalling pathways emanating from G-protein coupled receptors (Hamm, 1998). Upon activation they break up into two parts – α and βγ subunits. After its release from the trimeric complex, β-subunit containing WD40 domains mediates the recruitment of RACK1 to plasma membrane (Chen et al., 2004b). Gβγ-RACK1 complex then inhibits the activation of PLCβII and adenylyl cyclase II (Chen et al., 2004a).

2.10.2 RACK1-Src interaction

Src tyrosine kinase, involved in regulation of cell growth and differentiation, was also identified as RACK1 binding partner (Chang et al., 1998). It belongs to SFK superfamily and exhibit the same domain structures as described for Lck (Fig. 2.2). Moreover, using *in vitro* binding studies utilizing glutathione S-transferase (GST) fusion proteins, it has been shown that RACK1 is also able to interact with two other SFKs - Lck and Fyn (Chang et al., 1998). The interaction between RACK1 and SFKs is mediated by SH2 domains binding to the phosphotyrosines in sixth WD40 domain of RACK1 (Chang et al., 2001). Mutational analyses of HA-tagged RACK1 expressed in NIH3T3 cell line demonstrated importance of pY246 in sixth WD40 repeat for Src binding. In addition, both Y246 and Y228 can be phosphorylated by Src, suggesting that RACK1 as a substrate for Src (Chang et al., 2002). Tyrosine phosphatase PTPμ, has been also reported to bind to RACK1, but the physiological significance of this interaction remains to be elucidated (Mourton et al., 2001).

Interestingly, HA-RACK1 overexpression inhibits the activity of Src kinase and suppresses the growth of NIH3T3 cells by prolonging G_0/G_1 cell cycle phase (Chang et al., 1998). This is consistent with the demonstration that RACK1 overexpression regulates G_1/S progression by influencing cell cycle regulators in G_1 checkpoint (Mamidipudi et al., 2004). RACK1 downregulation via siRNA overcomes this block. Mechanism underlying this regulation relates to RACK1-mediated Src downregulation and interference with phosphorylation of Src substrates required for cell cycling (Mamidipudi et al., 2007; Miller et al., 2004). Thus, RACK1 mediated suppression of oncogenic Src shows critical function of this kinase in cell cycle arrest and points to the possibility to use this information for designing new methods for cancer treatment.

Besides above described interactions and mechanisms involving RACK1, several other interacting partners for RACK1 were described. Namely, PLCγ – functioning upstream of PKC signalling pathway; rasGAPs – responsible for the activation of small GTPases involved in various cellular processes including actin rearrangement; PDE4D5 – regulator of cAMP activation pathway to list just a few [reviewed in (McCahill et al., 2002; Schechtman and Mochly-Rosen, 2001)].

3 Materials and Methods

3.1 Materials

3.1.1 Mice

Three mice strains of C57BL/6 genetic background, six to eight week old males, were used: WT strain, OTII transgenic mice with T cell receptor transgene specific for OVA peptide (gift from Prof. Hořejší laboratory) (Barnden et al., 1998) and MHC class II–EGFP Knock-in mice (Boes et al., 2002). They were housed in pathogen-free animal facility at Institute of Molecular Genetics (Prague, Czech Republic).

3.1.2 Cell lines, cell preparation and culture

NIH3T3 fibroblast cell line, Lck infectants and HEK293T cell line were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 10% of inactivated fetal calf serum (FCS) and 100 Units of penicillin / 10µg streptomycin antibiotics (Sigma) per 1 ml of media.

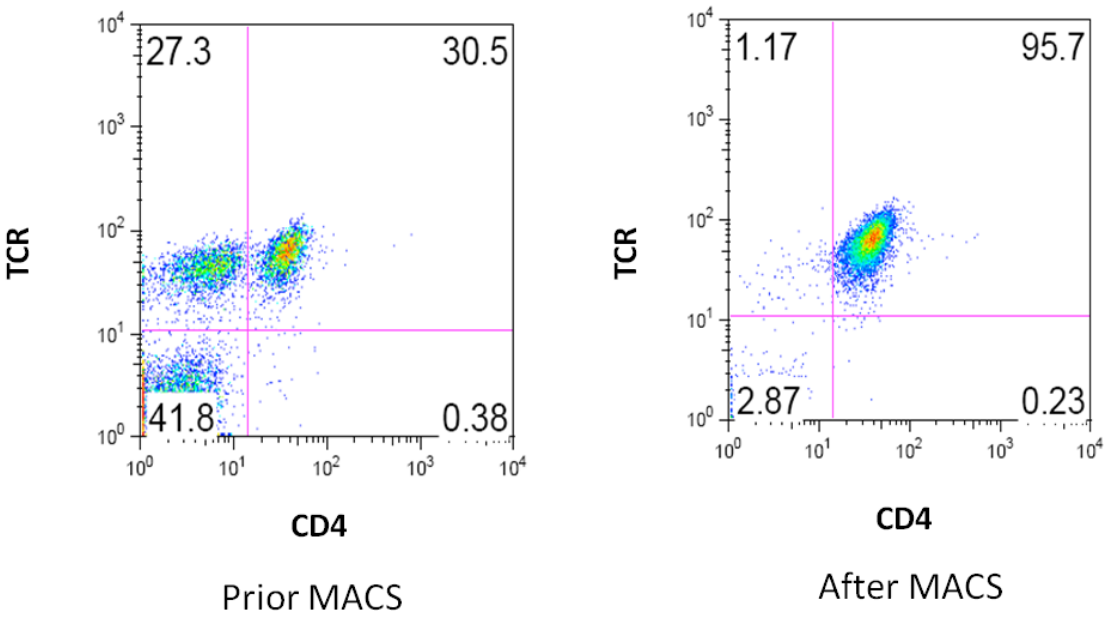


Figure 3.1 High enrichment efficiency of MACS CD4$^+$ T-cell separation. CD4$^+$ T-cells prior and after MACS-enrichment were stained with APC-conjugated anti-TCR (APC) and FITC-conjugated anti-CD4 and analyzed by FACS. Prior MACS, approximately 30% of total cells isolated from lymph nodes are CD4$^+$ T-cells (left panel). After MACS the purity of isolated CD4$^+$ T-cells increased up to 95% (right panel). Representative dot plots are selected from several independent experiments

Primary $CD4^+$ T-cells were obtained from axillary, brachial, inguinal and superficial cervical lymph nodes of 6-8 week old C57BL/6 mice males. Cell suspension was washed, counted and highly enriched $CD4^+$ T-cell were obtained by using MACS T-cell separation kit according to manufacturer's recommendation protocol (Milteney Biotech) (Fig. 3.1).

Bone marrow derived dendritic cells (BMDC) and macrophages (BMMF) from OTII transgenic mice were isolated from mice femur and tibia cavities. The cells were cultured for 6 days in RPMI medium supplemented with GM-CSF-containing supernatant produced by LUTZ cell line (final concentration was adjusted to 4%). After three days of cultivation, one half of the media was replenished and on day 6, the cells were pulsed with OVA antigen.

3.1.3 Antibodies

All antibodies used are listed in Table 1.

Name of Ab	**Source of Ab origin**	**Conjugate/purified**	**Source of Ab**
Primary Antibodies			
Anti-Lck	rabbit	Polyclonal	Gift from Prof. Hořejší laboratory
Anti-Fyn	rabbit	Polyclonal	
Anti-RACK1	mouse	Purified	Santa Cruz
Anti-RACK1 – N terminal	rabbit	Polyclonal	Sigma
Anti-αTubulin	rabbit	Polyclonal	GeneTex
Anti-Vimentin (VI-10)	mouse	AlexaFluor 488	Exbio
RHPM		Rhodamin-Phalloidin	Gift from Dr. Dráber
Anti-TCRCβ (H57)	hamster	Biotin	eBioscience
Anti-CD4 (GK1.5)	rat	Biotin	eBioscience
4G10 (pY)	mouse		Gift from Prof. Hořejší laboratory
12CA5 (HA)	mouse	Purified	
Secondary Antibodies - IHC			
Alexa Fluor® 488 goat anti-rabbit IgG		AlexaFluor 488	Invitrogen
Alexa Fluor® 555 goat anti-mouse IgG)		AlexaFluor 555	Invitrogen
Alexa Fluor® 647 goat anti-rabbit IgG		AlexaFluor 647	Invitrogen
Secondary Antibodies - WB			
GαM HRP		HRP	Biorad
GαM HRP True Blot		HRP	eBioscience
GαRa HRP		HRP	Biorad
Protein A HRP		HRP	Biorad
Cholera Toxin B subunit HRP		HRP	Sigma

Table 1. Primary and secondary antibodies and their specifications

3.1.4 cDNA vectors

Lck constructs were inserted into the murine stem cell virus (MSCV)-based internal ribosome entry site (IRES)-enhanced green fluorescent protein virus MigR1 (Pui et al., 1999), permitting the concurrent expression of a given gene and EGFP. Generation of retrovirus packaging cell lines and retrovirus stock as well as infection of NIH3T3 were performed as outlined elsewhere (Davidson et al., 1994; Leung et al., 1999).

3.2 Bacteria plasmid transformation

TOPO10 chemo-competent bacteria (E. coli, Invitrogen) were used for plasmid transformation. 1µg of plasmid DNA was added to 50µl-aliquot of bacteria, incubated for 20 minutes on ice and then subjected to heat shock in 42°C waterbath for 2 minutes. Sample was immediately returned on ice. 200µl of S.O.C. media (Invitrogen) was added and the bacteria was allowed to recover by incubation in 37°C incubator. After 30 minutes bacteria was plated out onto agar plates supplemented with appropriate antibiotic and incubated O/N in 37°C.

3.3 Plasmid isolation

Plasmids from transformed bacteria were isolated by Zyppy™ Plasmid Miniprep Kit II (Zymo research) according manufacture's protocol for low scale bacterial cultures (3 ml) and by Jetstar Plasmid Purification Midi Kit (Gemomed) for medium scale amounts (50 ml).

3.4 Flow cytometry (FACS) and cell sorting

FACS analysis was performed using Calibur or LSRII instruments, for sorting purposes we used FACSVantage SE instrument. Data were analysed using FlowJo statistic software. Cells were stained with direcly conjugated antibodies or sequentially, with primary then secondary Abs in appropriate combinations. The cells were washed and

analysed. Dead cell were excluded from analysis by addition of vital dye propidium iodide or Hoechst 33258.

3.5 Retroviral Cell infection

Transfection of HEK293 was conducted with Lipofectamine™ LTX and PLUS™ reagent (Invitrogen) and manufacture's protocol was followed with some small modifications. Briefly, $0{,}2x10^6$ of HEK293T cells were seeded into a 6 well plate and let to adhere overnight (O/N). On the second day, culture medium was replaced by Opti-MEM® I (Gibco) supplemented with 10% FCS without antibiotics. Transfection complexes were prepared by mixing 3µg of plasmid DNA (this consisted of 1µg plasmid DNA encoding Env gene, 1µ plasmid DNA encoding Gag and Pol retroviral genes, and finally 1µg of plasmid DNA coding the protein of interest) diluted in 500µl of Opti-MEM® I medium with 3µl of PLUS™ reagent. The mixture was incubated for 5 minutes at room temperature and 7,5µl Lipofectamine™ LTX reagent was added and incubating for 30 minutes at room temperature. Formed DNA complexes were then added dropwise to adhered HEK293T cells. After 6 hours of incubation the medium was exchanged for IMDM + 10% FCS and cells were incubated at 37°C for next 24-48 hours. In parallel, $0{,}2 \times 10^6$ NIH3T3 cells were seeded in 6-well plate and cultured O/N. Next day, in 24 hours post-transfection time point, virus particles-containing supernatant from HEK293T cells was transferred to NIH3T3 cells and polybrene reagent (Sigma) was added to a final concentration of 8µg/ml. Additional 2ml of media was added to HEK293T cells and newly produced viral particles were harvested and used for a second round of NIH3T3 infection after 24 hours. 48 hours after infection, the transfection efficiency was analysed by flow cytometry and positive clones were sorted out by cell FACS-sorting.

3.6 Transient lipofectamin transfection

$0{,}6 \times 10^6$ of NIH3T3 cells were seeded in 6-well plate to adhere O/N and were used for transfection assay using PLUS™ and Lipofectamine™ LTX reagents as described

above (chapter 3.6.) After 24 hours cells were harvested and used for immunoprecipitation.

3.7 SDS PAGE gel electrophoresis and Western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (WB) followed standard protocols described in Sambrook and Russel, 2001 (Vol. 3, App. 8, chapters 8.40 - 8.55). Briefly, samples were loaded into polyacrylamide gels (9% to 12,5%) and run in SDS running buffer (25mM Tris, 192mM glycin, 0,1% (w/v) SDS). Resolved proteins were transferred onto PVDF membrane (BioRad) by "wet" blotting in methanol transfer buffer (20mM Tris, 156mM Glycine, 20% MeOH). Membranes were washed in TBS-T buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 0,05% Tween) and blocked for 1 hour in 5% non-fat milk diluted in TBS-T or in 3% gelatine at 37°C (phospho blots). Blots were then incubated for 1 hour with primary antibodies diluted in blocking buffer (5% milk or 1% gelatine, respectively) followed by 5 washes in TBS-T (10 minutes each) and incubation with secondary antibodies conjugated to horseradish peroxidase (HRP). After 5washes (10 minutes each), blots were developed by incubation with the ECL substrate (Pierce) for 1 minute. Chemiluminiscent signals were visualized by exposure to X-ray films.

3.8 Immunoprecipitation

For immunoprecipitation purposes, antibodies directed against the protein of interest were bounded to Protein G Agarose beads (10µl bed volume/sample) (Roche) by incubation for 4 hours at 4°C. NIH3T3 cells or primary $CD4^+$ T-cell were lysed in hypotonic TNE lysis buffer (50mM Tris-HCl pH 8.0, 20mM EDTA pH 8.0, 1% NP-40, protease complete inhibitor cocktail (Roche), 20mM NaF, 0.2mM orthovandate-natrium), incubated on ice for 30 minutes and centrifuged at 4°C at 12000g for 10 minutes. Supernatant was transferred to antibody-pre-coated beads and incubated overnight at 4°C on rotation wheel. Next day beads were collected, washed five times with TNE buffer without protease inhibitors, boiled in reducing sample buffer at 100°C for 5-10 minutes and used for SDS-PAGE analysis

3.9 Antibody-mediated TCR/CD4 co-aggregation of primary $CD4^+$ T cells

Freshly isolated and highly enriched (~95% purity) naïve $CD4^+$ T-cell (AutoMACS T-cell separation kit, Miltenyi Biotec) were incubated with the mixture of biotin-conjugated antibodies (purchased from eBioscience) directed against TCR (H57 at 1 µg/ml) and anti-CD4 (GK1.5 at 0.3µg/ml) in 500µl of PBS for 30 minutes at 4°C. The cells were washes twice with cold PBS+3%FCS and then resuspended in 20 µl of PBS per sample. The cells were then pre-warmed for 1 minute in 37°C waterbath. TCR/CD4 co-aggregation was achieved by addition of streptavidin (Sigma) to the final concentration 50µg/ml, shortly vortexed and incubated at 37°C for indicated time. Activation was stopped by adding either ice-cold lysis buffer (immunoprecipitation), fixative (PFA) (microscopy) or loading sample buffer for (WB).

3.10 Formation of APC-CD4+T-cell conjugates

Preparation of conjugates for microscopic analysis of immunological synapse formation *in vitro* has been previously described (Lee et al., 2002). Briefly, BMDC and BMMF were prepared in parallel as described above (Methods section 3.1.2). Cells were then pulsed with $OVA_{323-339}$ peptide for two hours and TCR-transgenic $CD4^+$ primary T-cells isolated from OTII mice were admixed to APCs at 3:1 ratio. Formation of APC-T-cell conjugates was achieved by short spinning. Conjugates were incubated in serum-free IMDM medium at 37°C. The cells were fixed by 4% PFA for 15 minutes, stained with anti-RACK1 and anti-Lck directed Abs and used for the confocal microscopy analysis.

3.11 Gel filtration

Method is based on the previously described protocol (Cinek and Horejsi, 1992). Briefly, 5ml pipette tip closed with a small piece of glass wool was used as the column. It was filled with 2ml bed volume of Sepharose 4B beads (Sigma-Aldrich) and then equilibrated with two column volumes of cell lysis buffer. The cells were lysed in TKM-Brij58 lysis buffer (50mM Tris-HCl pH 8.0, 25mM KCl, 5mM $MgCl_2$, 1mM

EDTA pH 8.0, 0.5% Brij-58, protease complete inhibitor cocktail (Roche), 20mM NaF, 0.2mM orthovandatenatrium) for 30 minutes at 4°C and spun down for 1 minute at 800xg at 4°C. Supernatant in total volume of 1/10 of stationary bead volume (200 µl), was loaded on the top of the column and eluted with addition of cell lysis buffer. Of note, it took approximately 5 minutes to collect 1 fraction (200 µl) of the eluate. Totally 12 fractions were harvested. All steps were performed at 4°C. Figure 3.2 illustrates the elution profile of Lck in a typical experiment. WB analysis using antibody directed against the protein of choice was used to reveal its elution profile. Approximate molecular size marker for these fractions is indicated in Fig 4.10A.

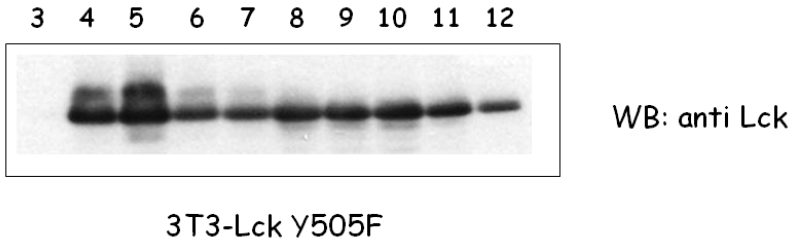


Figure 3.2 Lck fractionation by gel filtration. Cell lysates from 3T3-LckY505F infectants were fractionated according the procedure described in this section and probed with anti-Lck.

3.12 RAFT isolation

Isolation of lipid raft is based on their insolubility in mild non-ionic detergents and subsequent fractionation by ultracentrifugation in sucrose gradient where they float to the top fractions (Filipp et al., 2004). We used this method to assess LR distribution of proteins of interest present in fractions obtained by gel filtration. Pooled fractions (Fig.4.10A) were mixed 1:1 with 80% sucrose, loaded to the bottom of 5ml ultracentrifuge tube and successively overlayed with 4.3 ml of 36% and 0.2 ml 5% sucrose (Figure 3.3). Samples were centrifuged for 14-16 hours at 250000xg at 4°C. Ten fractions of 500µl volume were carefully collected from each tube. Usually, LR are enriched in top fractions (#1-3), while the bottom fractions (#8-10) contain soluble proteins or protein complexes.

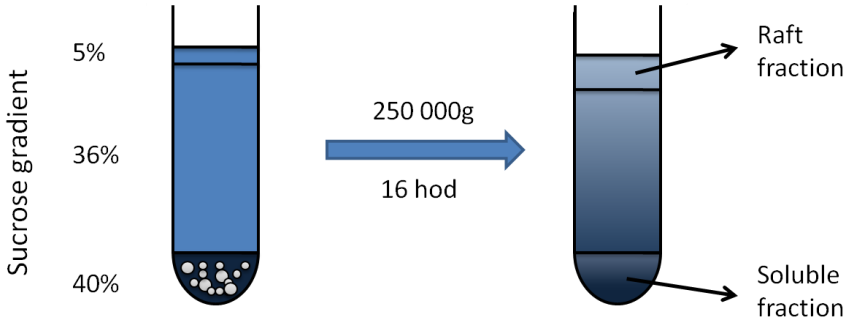


Figure 3.3 Isolation of lipid rafts by sucrose gradient floatation asssay. As lipid rafts float to the top of the gradient, their enrichment in top fractions is verified by binding of LR surrogate marker, ganglioside GM1, to cholera toxin B-subunit by WB analysis. WB analysis is also used to reveal the association of the protein of interest in regard to its distribution to LR.

3.13 Immunofluorescence microscopy

Immunofluorescence is used to visualize the localization and distribution of protein of interest within the cells. Visualization is achieved by binding of fluorochrome-conjugated antibodies or other substances which can bind to the protein of interest with high specificity and sensitivity. Protocol used for this study was adopted from the cell signalling website (www.cellsignal.com) with some modifications. Briefly, $3x10^4$ NIH3T3 cells were seeded on glass coverslips and let to adhere O/N. Next day the cells were washed and fixed for 15 minutes in 4% PFA. $CD4^+$ T-cells, upon their activativation, were immediately fixed in 4% PFA for 15 minutes and then places on coverslips by cytospin centrifugation (Hettich). Then, the following procedure was implied for all cell types. After PFA fixations, the cells were washed three times with PBS and permeabilized with ice-cold methanol for 10 minutes in -20°C (methanol step had to be omitted when phalloidine was used to stain actin cytoskeleton). The cells were blocked for one hour with PBS containing 0,3% Triton X-100 (PBT) with addition of 2,5% FCS and 2,5% BSA and then incubated with primary antibody diluted in PBT for one hour. Samples were washes 3 times in PBS (5 minutes each) and then incubated with secondary antibody for one hour. After 3 PBS washes, the coverslips were mounted using Vectashield containing DAPI (Vector). To avoid undesirable slippage, coverslips were fixed to glass by colourless nail-polish. Samples were analyzed by confocal microscopy (Leica DMI6000 with TCS SP5 AOBS Tandem) with HCX PL

APO 40x/1,25-0,75 Oil CS UV and HCX PL APO 63x/1,40-0,60 Oil, Lbd Blue CS objectives. A statistical analysis was done with LAS AF software program.

3.14 RNA isolation and RT-PCR

Reverse Transcription Polymerase chain reaction (RT-PCR) is a method where transcription of RNA to cDNA is followed by cDNA amplification. It is used for expression profiling or assessment of gene expression in a given sample.

Total RNA isolated from $0.5x10^6$ primary $CD4^+$ T-cells was isolated by QIAGEN RNeasy® Plus Microkit (Qiagen) according to manufacturer's protocol. Reverse transcriptase (Superscript II, Invitrogen) was used to convert RNA to cDNA in three steps by successively adding reaction mixtures to the RNA template as highlighted below.

	Step 1.		**Step 2.**		**Step 3.**	
	hexamers	- 1µl	RT buffer	- 4µl		
	dNTP	- 1µl	DTT	- 2µl		
	RNA	- x µl (1µg)	RNAout	- 1µl		
	H_2O	to total 12µl	H_2O	- 0,5µl	RT-SuperscriptII	- 0,5µl
Time/°C	5 min / 65°C		2min / 25°C		10 min / 25°C 60 min / 42°C	

Retrieved cDNA was amplified by XP cycler instrument using the reaction mixture described below. Amplification conditions are also outlined.

dNTP	0,2 µl
Taq Pol. Buffer	2 µl
Forward primer	1 µl
Reverse primer	1 µl
Taq polymerase	1 µl
cDNA (sample)	1 µl
H_2O	13,8 µl
Total volume	20 µl

Cycler program:

temperature	time	
94°C	120s	
94°C	30s	36
54°C	30s	cycles
72°C	60s	

gene	Forward primer	Reverse primer
RACK1	tctgcaagtacacggtccag	gagacgatgatagggttgctg
MORG-1	ccggtgcaaacactagatga	taggtcatagcgcctcacg
EED	aaattccacccacgagacc	ggatattccataaccgtaaagca

Table 2: List of Used Primers. Primers were designed by Roche Universal Probe Library (ProbeFinder version 2.44 - https://www.roche-applied-science.com/sis /rtpcr/upl/index.jsp)

3.15 Agarose gel electrophoresis

DNA samples were mixed with sample loading buffer containing crystal violet dye and loaded onto 0,5–2% agarose gels with ethidium bromide (1μg/ml). DNA was run in TBE buffer and resolved by applying a constant voltage. DNA Ladder Mix GeneRuler™ (Fermentas) was used as the standard molecular weight marker.

4 Results

4.1 General considerations

As described in the section 2.8.1, RACK1 was identified in our screen as a candidate phosphoprotein involved in the regulation of Lck translocation into LR. We have considered RACK1 as a good match for this role for the following four reasons: (i) capacity of RACK1 to interact with multiple binding partners (McCahill et al., 2002) suggesting that this protein can form heteromeric protein complexes; (ii) RACK1 is directly involved in the process of intracellular redistribution of PKC kinases during cell activation (Ron et al., 1999) giving a precedence for its active participation in membrane translocation events; (iii) RACK1 is a physiological substrate for Src kinase with which it can interact through Src-SH2 domain (Chang et al., 2001); and lastly (iv) RACK1 affects the kinase activity of Lck *in vitro* (Chang et al., 1998) suggesting a physiological interplay between these two molecules.

Our initial goal was to verify whether Lck is able to form complexes with RACK1 in Lck-C-terminal-dependent fashion and thus confirm results from our original screening strategy (chapter 2.9). Towards this end, NIH3T3 fibroblast cell line co-expressing defined variants of Lck together with an endogenous or ectopically expressed RACK1 protein were used for co-immunoprecipitation experiments.

4.2 Preparation of NIH3T3 Lck mutants

First, NIH3T3 cells (3T3) expressing the wild type Lck (WT), the constitutively active Y50F-Lck, the kinase inactive Y394F-Lck, Lck variants with inactivating single point mutation in either SH2 (R154K) or SH3 (W97K) domain introduced on Y505F-Lck template and the C-terminal truncate of the last 11 amino acid residues (ΔC11) were prepared as previously described (Filipp et al, 2008) (Fig. 4.1A). Mutated Lck cDNA were introduced into cells by retroviral infection using the biscistronic vector Migr1 permitting the concurrent expression of a given Lck variants and EGFP. Infected cells (3T3-Lck) were sorted out based on the expression of EGFP (Fig. 4.1B) and maintained in cell cultures. Protein expression of Lck and RACK1 (Fig. 1C) is comparable among

all FACS-sorted 3T3-Lck infectants and were used for further analyses. It is of note that 3T3 cells do not express endogenous Lck.

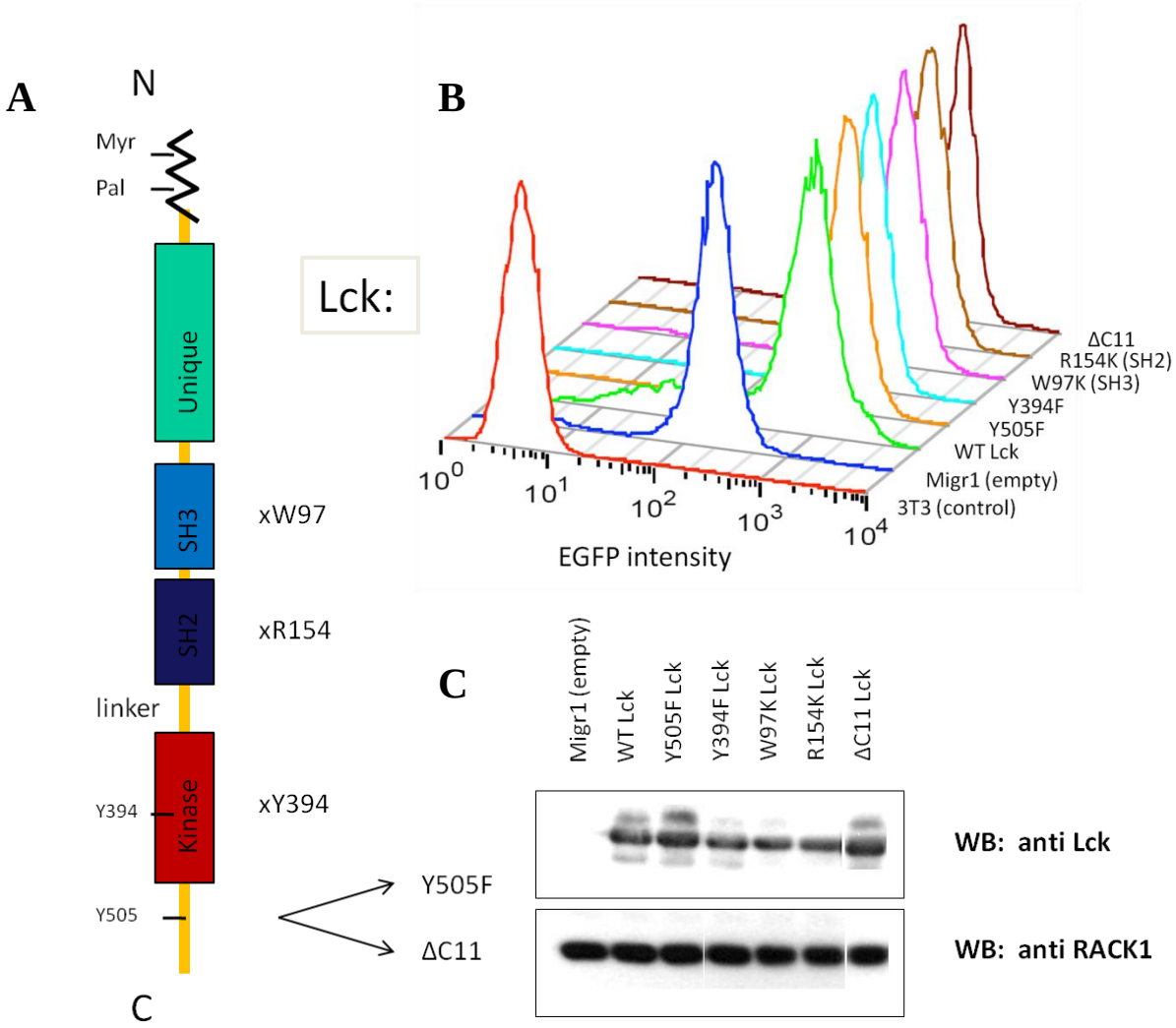


Figure 4.1 Preparation of NIH3T3 fibroblast cell lines expressing comparable levels of various Lck mutants. **A.** Lck domain structure with indicated point mutations/deletion affecting the function of SH3, SH2 and catalytic domains and the regulatory C-terminal region is depicted. **B.** Histogram showing comparable intensity of EGFP expression among Lck infectants measured by flow cytometry. **C.** Western blot analysis performed on total cell lysates revealed comparable protein levels of Lck and RACK1 among indicated Lck infectants.

4.3 Kinase active Lck complexes with RACK1

To determine whether the presence of tyrosine-phosphorylated RACK1 in the 3T3 cells co-expressing kinase active Y505F-Lck (see Fig. 2.7) correlates with the ability of Lck to interact with RACK1, hemagglutinine-tagged RACK1 (HA-RACK) was co-expressed in 3T3 cells with empty vector MIGR1, or MIGR1 encoding either WT or

Y50F-Lck. Cells were lysed (TNE buffer, 1% NP-40) and immunoprecipitated HA-RACK1 was probed for Lck, HA-RACK1 and phosphotyrosine (pY) content. As illustrated in Fig. 4.2, HA-RACK1 is readily expressed in all 3T3 infectants used (bottom panel). Probing anti-HA-RACK1 immunoprecipitate with anti-Lck antibody revealed that Y505F-Lck, but not WT-Lck, efficiently co-immunoprecipitates with HA-RACK1 (Fig 4.2, top panel). In addition RACK1 complexed with Y505Lck is also tyrosine phosphorylated (Fig. 4.2, middle panel). These data are consistent with data obtained from 2D-gel analysis and suggest that complex formation between kinase active Lck and RACK1 correlates with RACK1 phosphorylation.

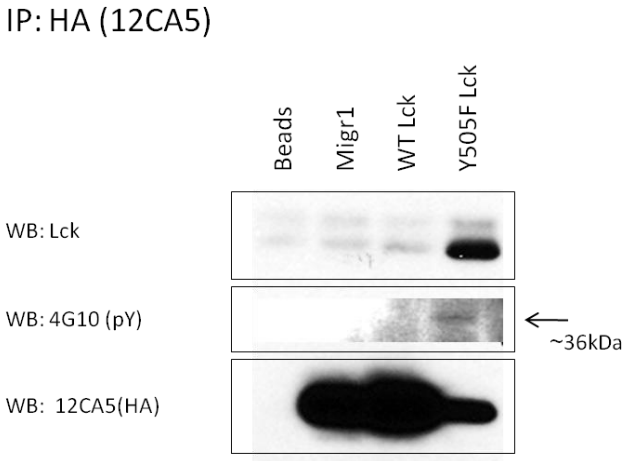


Figure 4.2 Lck is able to interact with RACK1. 3T3 cells infected with WT or Y505F Lck were transfected with HA-tagged RACK1. RACK1 was immunoprecipitated with anti-HA antibody and equal aliquots were probed for Lck (upper panel), phosphotyrosine content with 4G10 mAb (middle panel) and RACK1-HA with anti-HA 12CA5 mAb (bottom panel). Constitutive active form of Lck (Y505F) but not WT-Lck, co-immunoprecipitates with overexpressed HA-RACK1 which is tyrosine-phosphorylated.

4.3.1 Structure-function analysis of Lck-RACK1 interaction

Next, we examined whether ectopically expressed Lck also interacts with endogenous RACK1 and if yes, whether this interaction is dependent on certain Lck domains or functional segments. Endogenous RACK1 was immunoprecipitated with anti-RACK1 polyclonal rabbit antibody from 3T3 Lck infectants and aliquots of immunoprecipitate were blotted for Lck (Fig. 4.3A, upper panel) and RACK1 (Fig. 4.3A, bottom panel).

We found that Y505F and Y394F-Lck interact comparably with endogenous RACK1 and this interaction is slightly diminished in cells expressing the WT-Lck. However, a significant reduction in the formation of Lck-RACK1 complexes is observed using SH2 and SH3 domain mutants as well as the C-terminal Lck truncate (Fig. 4.3A, upper panel). Quantification analysis revealed that Lck-RACK1 interaction was ~2-5 fold lower compared to that between Y505F and or Y394F-Lck and RACK1. It suggests that functional modular domains and the C-terminal tail of Lck are prerequisites for Lck-RACK1 complex formation.

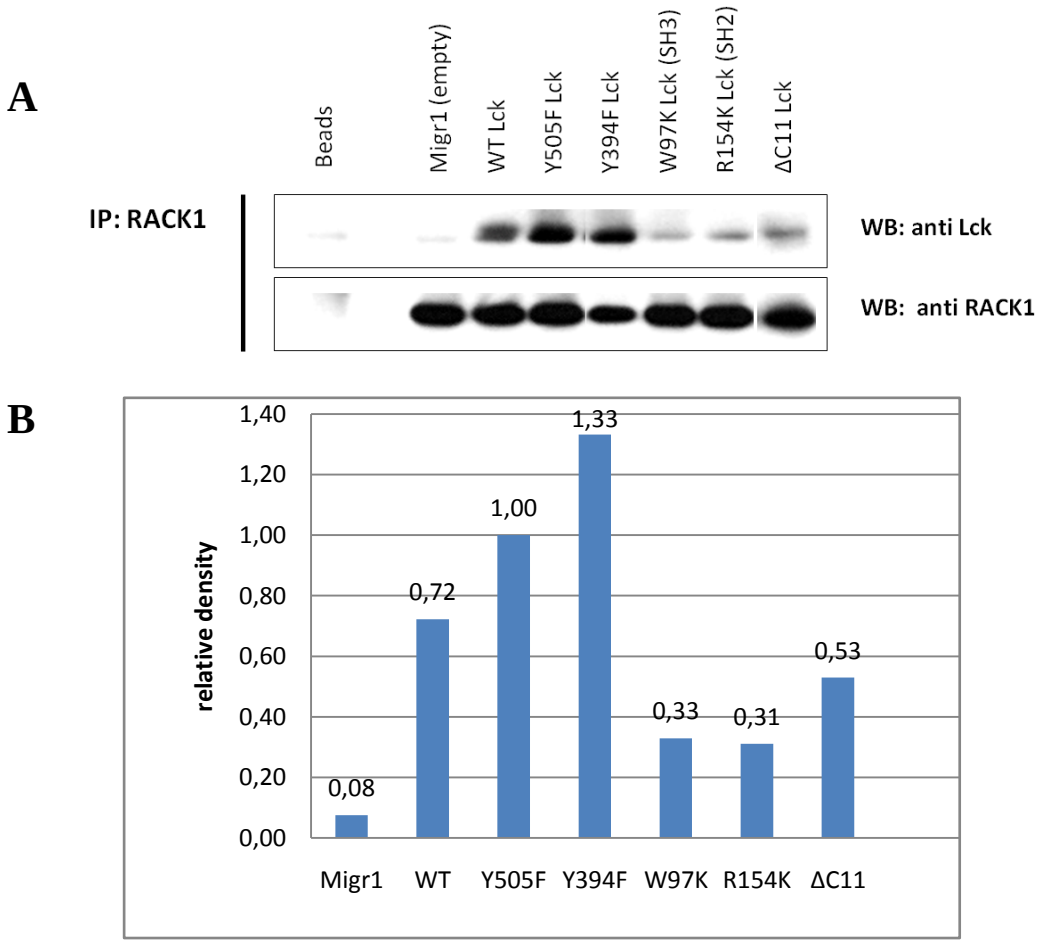


Figure 3. RACK1-Lck interaction depends on functional SH3, SH2 and the C-terminal domains. A. Endogenously expressed RACK1 was immunoprecipitated from indicated 3T3-Lck infectants and blotted with anti-Lck (upper panel) and anti-RACK1 (bottom panel) antibodies. **B)** Bar graph shows the relative amount of Lck immunoprecipitated with RACK1 measured by normalization to the total amount of immunoprecipitated RACK1. As an arbitrary reference value was assigned Y505F signal (=1). Blots and graph are representative of three independent experiments.

4.3.2 Lck-RACK1 complex formation in primary CD4$^+$ T-cells

To verify that Lck-RACK1 interaction is not an artefact of fibroblast model system, we next determined whether this interaction is detectable under more relevant physiological circumstances. We first assess whether RACK1 is expressed in primary lymph node CD4$^+$ T-cells. Total mRNA isolated from highly enriched CD4$^+$ T-cells obtained by MACS separation (~95% purity) and further purified by FACS to 99.3% (Fig. 4.4A) positive CD4$^+$ population was used for RT-PCR analysis. As illustrated in Fig. 4.4B, RACK1 is expressed in non-activated CD4$^+$ T-cells.

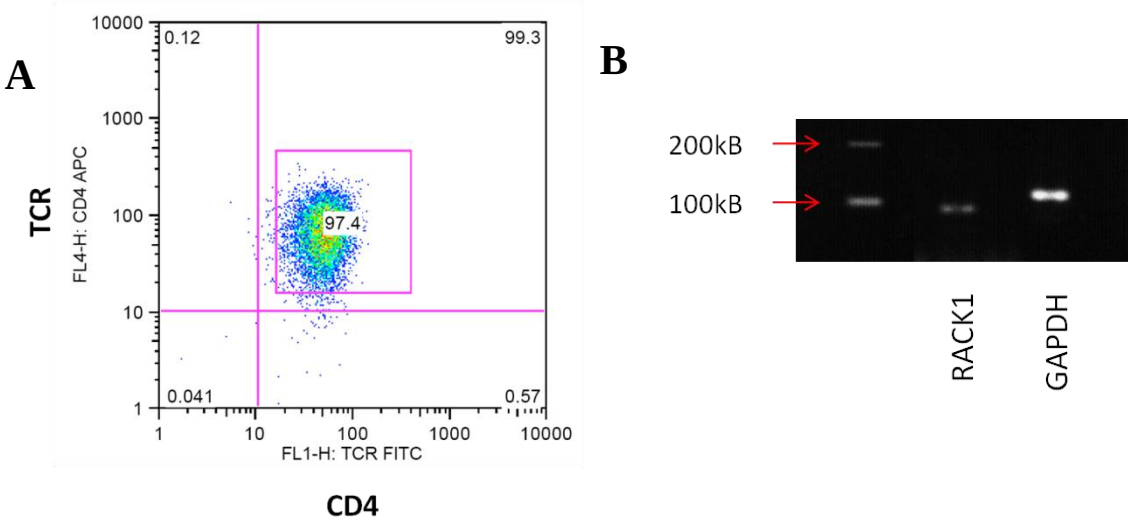


Figure 4.4 RACK1 is expressed in murine CD4$^+$ T-cells. A. Phenotypic profile of CD4$^+$ T-cells obtained by MACS separation followed by FACS sorting. (Red rectangle = sorting gate). **B.** RT-PCR was performed on mRNA isolated from CD4$^+$ lymph node T-cells according the protocol and conditions described in the section Material and Methods (3.14).

To determine whether Lck-RACK1 interaction in CD4$^+$ T-cells occurs constitutively or not, or whether this interaction is activation-inducible, we utilized the recently established model of T-cell activation enabling to study the kinetics of interaction between two interacting proteins during the first seconds after TCR/CD4 engagement. Specifically, primary CD4$^+$ T-cells were pre-coated with biotin-conjugated antibodies specific for TCRCβ and CD4 and activated by the addition of streptavidin. Antibody mediated co-aggregation of TCR and CD4 induces robust global tyrosine phosphorylation of cellular substrates throughout the co-aggregation time course analysed (Fig 4.5).

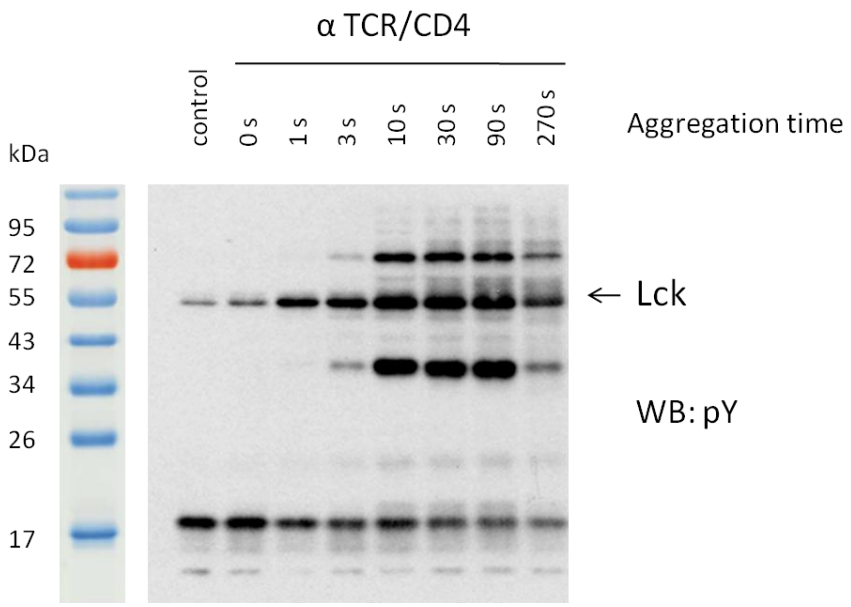


Figure 4.5 Time dependent phosphotyrosine status upon T-cell activation via co-aggregation: Freshly isolated primary $CD4^+$ lymph node T-cells were pre-coated, or not (control), with biotinylated anti-TCR (H57) and anti-CD4 (GK1.5) mAbs (αTCR/CD4) and co-aggregated, or not (0 sec), with addition of streptavidin for time indicated. Phosphotyrosine content of cell lysates derived from $0.5x10^6$ cells/sample was probed with 4G10 antibody. One representative blot from several independent experiment is shown.

Using this method, T-cell were pre-coated or not (control sample) with biotin-conjugated antibodies specific for TCRCβ and CD4 and activated, or not (0 sec), for 10, 30, 90 and 270 second. Activation was stopped by directly adding a cold TNE lysis buffer, cell lysates were immunoprecipitated using anti-RACK1 antibody and aliquots were probed for Lck and RACK1. As illustrated in Fig. 4.6, RACK1-Lck complexes were not detected in control sample. However, relatively robust Lck-RACK1 co-immunoprecipitation signal was observed in antibody pre-coated but not co-aggregated (0 sec) sample. Interestingly, intensity of this signal decreased to ~30% of its value over the ensuing 270 seconds after TCR/CD4 co-aggregation. In sum, this data suggests that in naïve non-activated $CD4^+$ T-cells Lck and RACK1 are not associated. Simultaneous, yet independent engagement of TCR and CD4 receptors induces Lck-RACK1 complex formation even though T-cells activation per se, as measured by induction of global tyrosine phosphorylation, is for this process dispensable.

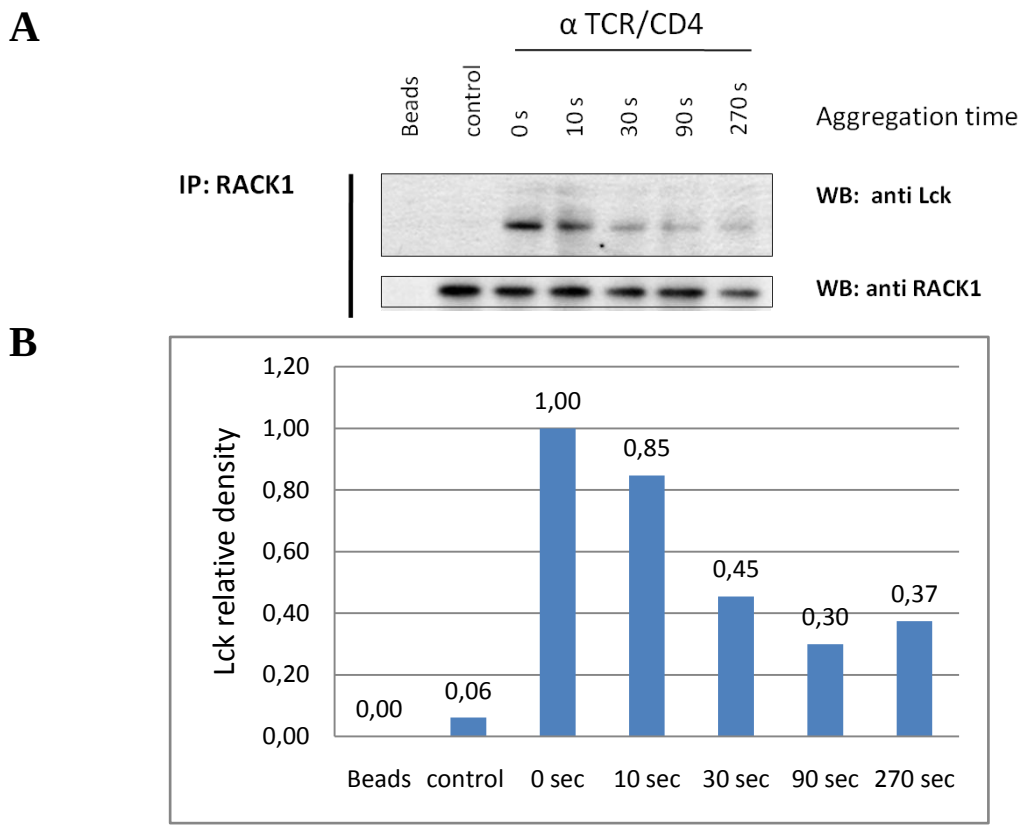
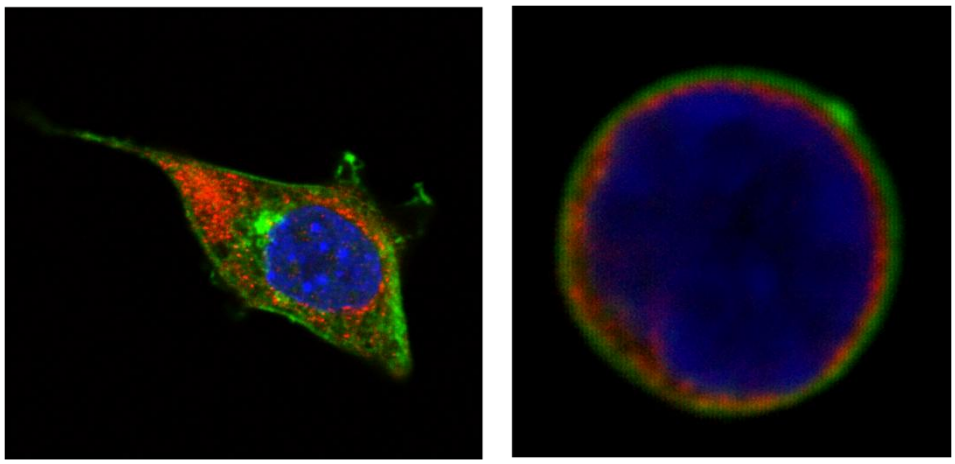


Figure 4.6 Antibody-mediated engagement of TCR/CD4 receptors induces Lck-RACK1 complex formation. A. Freshly isolated primary $CD4^+$ lymph node T-cells were pre-coated, or not (control), with biotinylated anti-TCR (H57) and antti-CD4 (GK1.5) mAbs (αTCR/CD4) and co-aggregated, or not (0 sec), with addition of streptavidin for time indicated (in sec). $2x10^6$ cells per each time point were immunoprecipitated with anti-RACK1 and blotted against Lck (upper panel) and RACK1 (bottom panel). **B.** Bar graph shows the relative amount of Lck immunoprecipitated with RACK1 measured by normalization to the total amount of immunoprecipitated RACK1. The amount of Lck co-immunoprecipitated with RACK1 in cells pre-coated with anti-TCR/CD4 but not aggregated was assigned an arbitrary reference value of "1". The figure is representative of two independent experiments.

4.4 Common redistribution dynamics of Lck and RACK1 in activated T-cells

In previous result sections we presented biochemical data which demonstrate the physical interaction between Lck and RACK1 in both fibroblast 3T3 cells and primary $CD4^+$ T-cells. Next, we wanted to assess whether this interaction can be visualized microscopically in form of Lck-RACK1 co-localization. To gain some insight in the cellular distribution of both proteins, 3T3 cells and purified primary lymph node $CD4^+$ T-cells were stained with anti-Lck and anti-RACK1 and inspected by confocal microscopy. As illustrated in Fig. 4.7, in 3T3 cells, Y50F-Lck (green) preferentially localizes at the cell membrane while RACK1 (red) predominantly in cytoplasm where it

forms speckle-like structures. No obvious co-localization was observed. Moreover, 3T3 cells ectopically expressing either W97K-Lck SH3 or ΔC11-Lck mutant, which were unable to efficiently bind RACK1, showed similar distribution of these two proteins as 3T3 Y505F-Lck infectant (data not shown). In naïve unstimulated (control) $CD4^+$ T-cells, RACK1 fills up the constrained space between the nucleus and plasma membrane and Lck localizes exclusively to the plasma membrane. Similar to the situation in 3T3 cells, Lck-RACK1 co-localization was undetectable.



NIH3T3 – Y505F-Lck **$CD4^+$ T-cell**

Figure 4.7 Distribution of Lck and RACK1 in NIH3T3 cells and freshly isolated primary CD4+ lymph node T-cells. Cells were fixed in 4% PFA and probed with anti-Lck (green) and anti-RACK1 (red). One representative sample for each cell type is shown. Nuclei are stained with DAPI (blue). (x400 zoom).

To test whether in primary $CD4^+$ T-cells the co-localization of Lck and RACK1 is depend on TCR-CD4-co-aggregregation, the same "antibody-mediated activation" protocol was implied (section 4.3.2 and figure 4.6). After activation, T-cells were fixed, stained for Lck (green) and RACK1 (red) and analysed by confocal microscopy.

It is of note that αTCR (H57) and αCD4 (GK1.5) are of hamster and rat origin respectively and hence do not cross-react with secondary antibodies used in this assay. In control cells, non-precoated with Abs against TCR, CD4, Lck and RACK1 were evenly distributed within the cell membrane and cytoplasm, respectively (Fig. 4.8A). Upon TCR/CD4 co-aggregation, we observed a time-dependent co-redistribution of Lck and RACK1 into a restricted subcellular compartment in approximately 50% of all cells

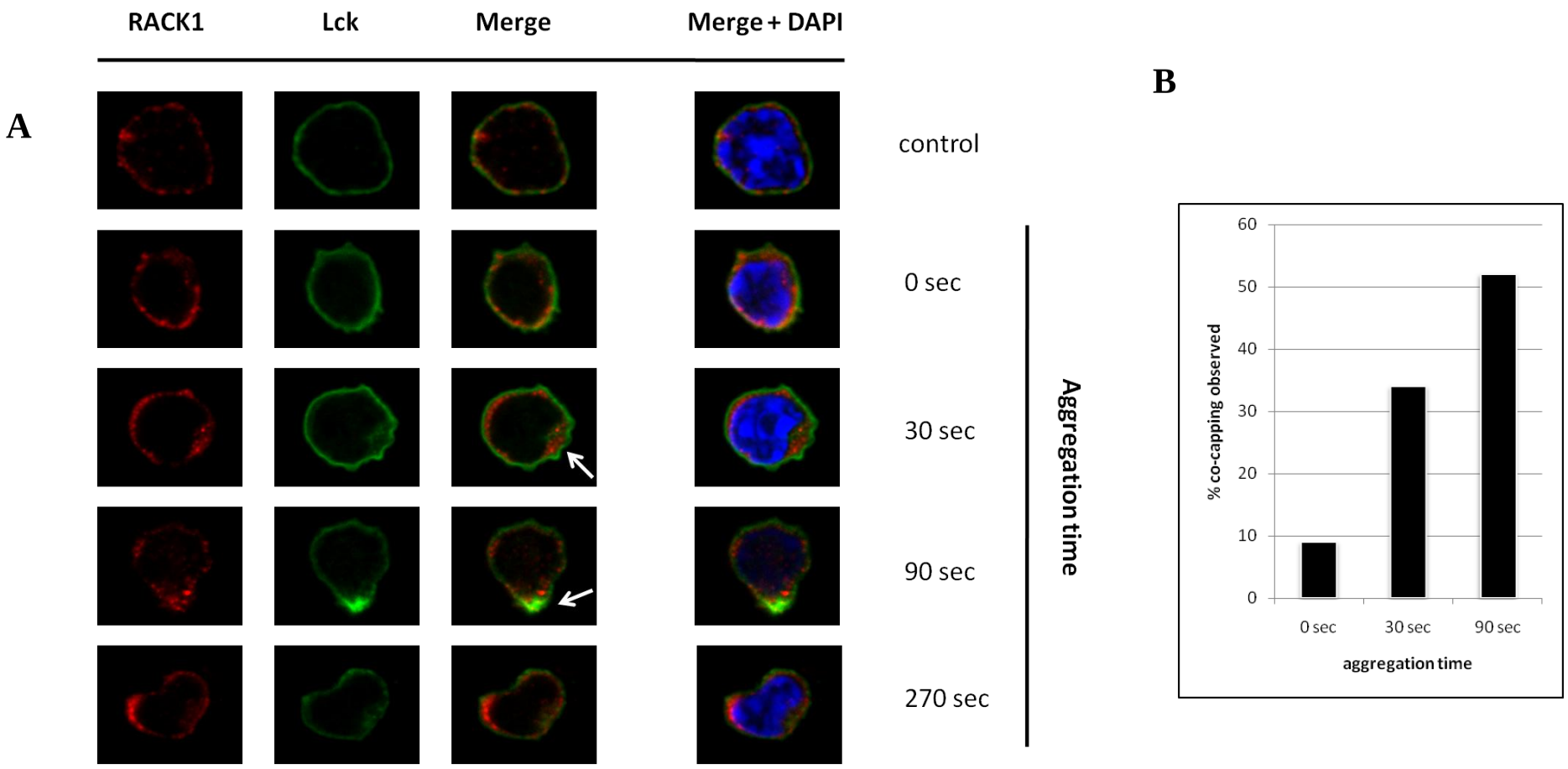


Figure 4.8 Lck and RACK1 co-redistribute upon T-cell activation. A. Primary CD4+ T-cells pre-coated or not (control) with biotinylated anti-TCR and anti-CD4 antibodies were activated or not (0 sec) by addition of streptavidin for time indicated and immediately fixed in 4% PFA. Cells were then mounted on cover slips using a cytospin centrifugation technique and probed with anti-Lck (green), and anti-RACK1 (red). Nuclei are stained with DAPI (blue). One representative sample per each time point and the control is shown. Arrows point to forming "co-capping" structures. **B.** At least 50 cells from each time point were microscopically analysed for the presence of "co-capping" structures. Bar graph shows the percentage of cells observed to be positive. (x400x zoom).

analysed (Fig. 4.8B). Formation of similar microscopic structures after antibody-mediated crosslinking of T-cell receptor(s) called “capping” has been already described (Kupfer and Singer, 1988). Co-capping of Lck and RACK1 peaks at 30 to 90 seconds after TCR/CD4 co-aggregation (Fig. 4.8A). However, three minutes after activation, Lck distribution returned to its initial, pre-activation state (nearly even membrane distribution), while RACK1 was retained clustered (Fig. 4.8A, bottom images).

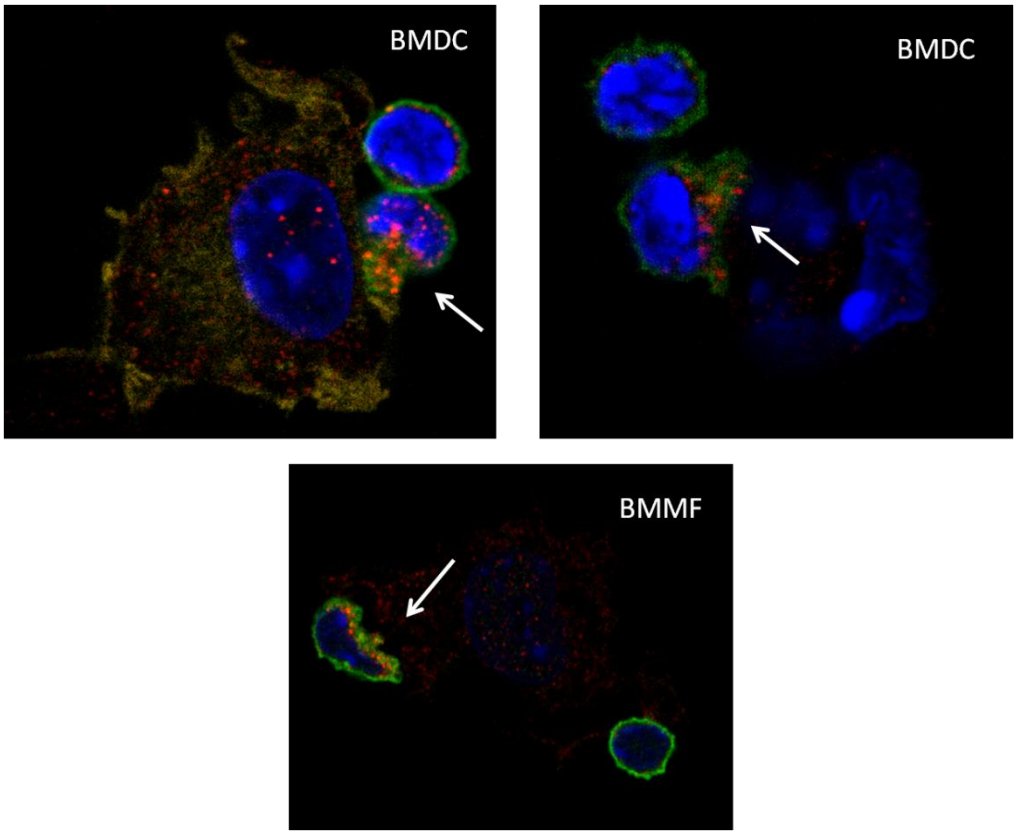


Figure 4.9 Lck and RACK1 co-redistribute into forming immunological synapse (IS) during early phases of $CD4^+$ T-cell/APC interaction. Bone marrow derived-dendritic cells (BMDC) and -macrophages (BMMF) generated from MHCII-EGFP knock-in mice were pulsed with OVA peptide and mixed with lymph node-derived $CD4^+$ T-cells isolated from OTII transgenic mice (with TCR specific for OVA peptide). After approximately 2-3 minutes, T-cell/APC conjugates were seeded on a cover slip, fixed with 4% PFA and probed with anti-Lck (green), anti-RACK1 (red). MHCII-EGFP molecules expressed on the surface of APCs are in yellow (it is of note however, that this expression is very weak on BMMF and some BMDC cells). Arrows point to forming IS. Representative images are shown. Nuclei are stained with DAPI (blue). (x400 zoom).

Redistribution of Lck and RACK1 was also examined in more physiological conditions, during early phases of immunological synapse formation. Bone marrow-derived

dendritic cells and macrophages pulsed with OVA peptide were mixed with transgenic T-cells specific for OVA peptide, and allowed to form conjugates. The cells were then fixed and immunostained for Lck (green) and RACK1 (red). Microscopic analysis showed that Lck and RACK1 concomitantly translocated to IS at early phases of its formation (2-3 minutes) (Fig. 4.9). Taken together, these data (Fig. 4.8 and 4.9) suggest that T-cell activation induces a rapid, cooperative and membrane-directed movement of Lck and RACK1. This co-redistribution pattern further strengthened the original suggestion that RACK1 is a member of translocation machinery regulating the distrubution of membrane Lck.

4.5 Subcellular distribution of Lck complexes

As reported previously, Lck prepared from unstimulated resting $CD4^+$ T-cells by solubilization with non-ionic detergent Brij-58 is highly enriched in soluble cellular fractions. Upon activation, 10-30% of Lck from this soluble pool is found translocated to lipid rafts (Filipp et al, 2003). As RACK1 is likely to be involved in this translocation process, we decided to characterize this translocating pool of Lck in greater detail. The main idea was to fractionate cellular components, first according to their size, and couple this step to their subsequent LR fractionation. This approach was expected to provide an insight into a size-dependent distribution of Lck complexes to LR. First, we adopted a gel filtration chromatography protocol (size exclusion chromatography) (Cinek and Horejsi, 1992) to identify size of Lck complexes over its elution profile (Fig. 4.10A, top 3rd panel). Results demonstrate the existence of Lck-associated complexes with significant differences in their size, ranging approximately between 10-100 to more than 1000 kDa (Fig.4.10A, size marker on the top). As illustrated in fig. 4.10A top two panels, LR-surrogate marker GM1 and LR-resident Fyn kinase co-fractionate with high molecular weight fractions (HMWFs) #4-5.

To assess the LR-distribution of Lck-associated molecular complexes, fraction number 4+5, 6+7, 8+9 and 9+10 were pooled and subjected to sucrose gradient floatation assay (SGFA) (Fig.4.10B). Result shows that only a small portion of Lck from HMWF #4-5 partitions to LR (~5-20%) while Lck from intermediate and low MW fractions (#6-12) is detected in soluble fractions exclusively. Fyn, which associated exclusively with HMWF #4-5 (Fig.4.10A 2nd top panel), is detected only in LR fractions (Fig.4.10B,

bottom panel). Interestingly, RACK1, which is preferentially associated with HMWFs #4-6 (Fig 4.10A, bottom panel), is undetectable in any of the fractions after SGFA (data not shown).

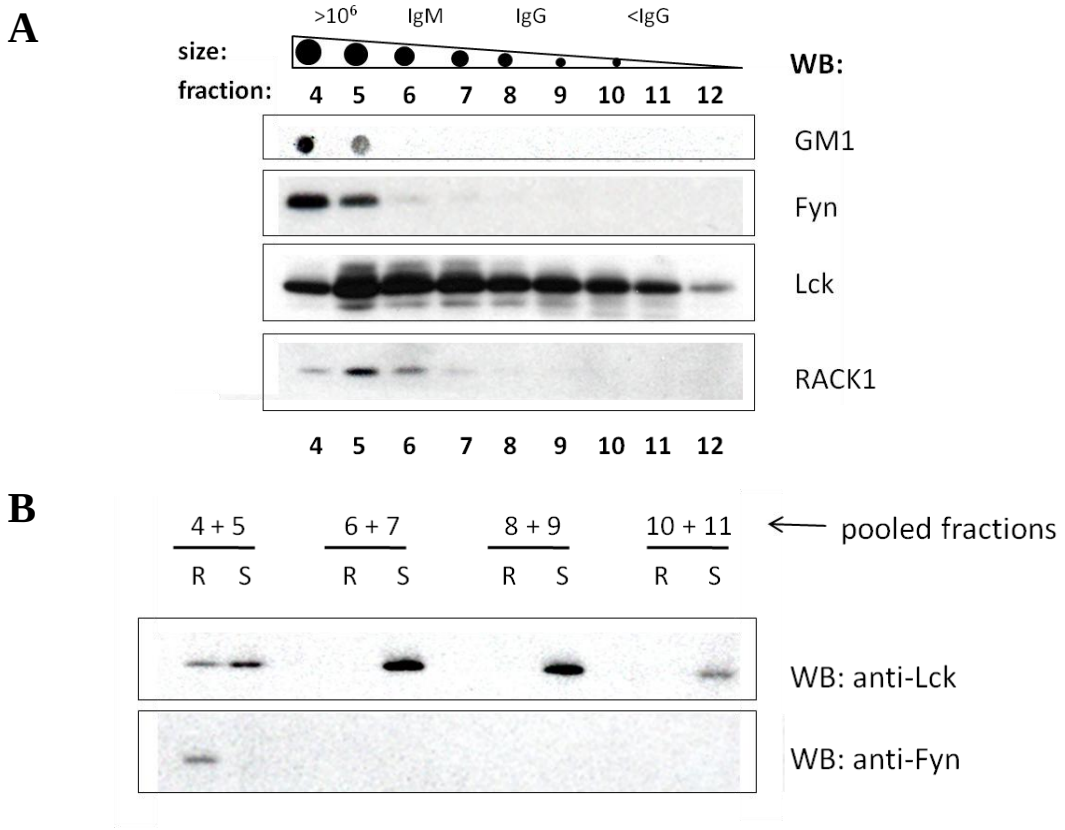


Figure 4.10 Distribution of high molecular weight complexes-associated Lck in lipid rafts. **A.** Fractionation of $CD4^+$ T-cells lysed in TKM+0,5% Brij58 buffer using a Sephadex gel filtration chromatography. Lck-containing fractions (4-12) were resolved on 9% SDS-PAGE gel, transferred onto PVDF membrane and probed with cholera toxin B subunit-HRP detecting lipid raft marker GM1 (upper panel), anti-Fyn (2^{nd} from the top panel), anti-Lck (3^{rd} from the top panel) and anti-RACK1 (bottom panel). Approximate molecular weight size marker is shown on the top. **B.** Sucrose gradient flotation assay of pooled fractions (as indicated) obtained from gel filtration chromatography. Lipid raft-containing fraction #1 (R) and soluble protein enriched fraction #10 (S) were probed with anti-Lck (upper panel) and anti-Fyn (bottom panel). Blots are representative of three independent experiments.

4.6 Lck associated with HMW-complexes translocates to lipid rafts.

To determine which type of Lck-associated complexes translocate to LR upon TCR/CD4-mediated activation, fractions from $CD4^+$ T-cells activated or not for 30 seconds by TCR/CD4 co-aggregation and prepared by gel filtration, were pooled (#4-5 and #6-7) and were subjected to SGFA as described in the previous section. As illustrated in Fig. 11A, and consistent with previous experiments, only a small

proportion (~10%) of HMWF-associated Lck (#4-5) from unstimulated T-cells portions to LR (Fig. 4.11A, top panel). Antibody-mediated activation induced translocation of more than 50% of Lck from HMWFs (#4-5) to LR. On the other side distribution of Lck from intermediate molecular weight fractions (#6-7) remained unchanged, and Lck was detected exclusively in soluble fractions. Taken together, this data strongly suggest that upon activation, only the pool of HMWF-associated Lck is targeted to LR. Moreover it

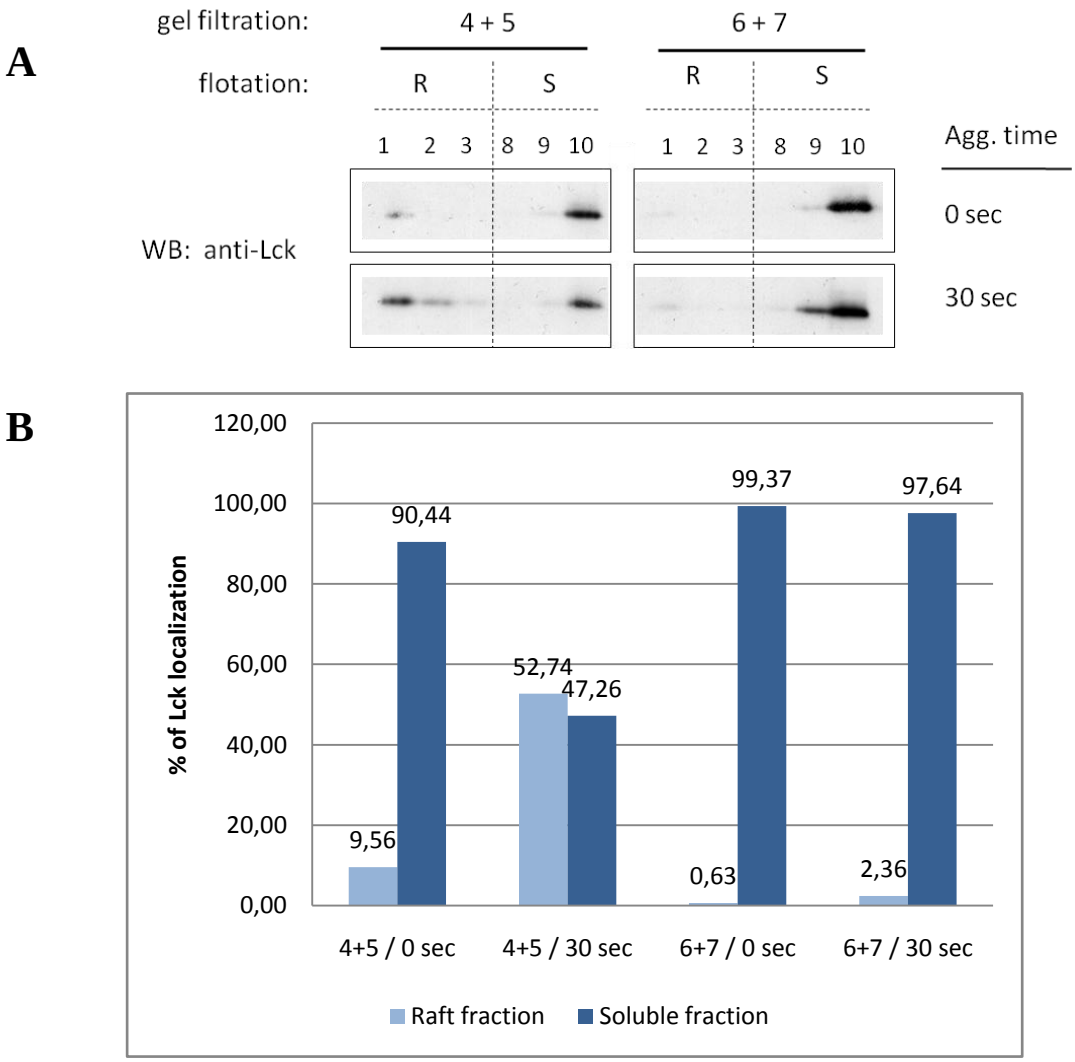


Figure 4.11 High molecular weight-associated Lck translocates to lipid rafts upon T-cell activation. A. Primary CD4+ T-cells, pre-coated with biotinylated anti-TCR and anti-CD4 antibodies, were activated, or not (0 sec) by addition of streptavidin for 30 seconds and immediately lysed in a cold TKM+0,5% Brij58 buffer. High (4-5) and intermediate (6-7) molecular weight fractions prepared by gel filtration were subsequently subjected to sucrose gradient flotation assay. Equal aliquots of LR fractions (1-3, R) and soluble fractions (8-10, S) were probed with anti-Lck. **B.** Bar graph represents the percentage distribution of Lck in lipid rafts in high and intermediate molecular weight fractions before and after antibody-mediated activation of T-cells as measured by Western blot signal densitometry. Blots are representative of 3 independent experiments.

indicates that RACK1 which is able to inducibly interact with Lck (Fig. 4.6) and co-fractionates with HMWFs, can participate in Lck translocation process.

4.7 RACK1 co-localizes with microtubular network

Cytoskeleton plays a critical role in T-cell activation (Gomez and Billadeau, 2008). To investigate whether RACK1 can be involved in Lck translocation through its binding to cytoskeleton component(s), we assessed the capacity of RACK1 to co-localize with actin, vimentin or microtubulin cytoskeletal components. NIH3T3 cells were fixed with PFA and immunostained with anti-RACK1 in combination with antibody directed against the one of three above mentioned cytoskeleton components – actin, vimentin and α-tubulin. Data obtained suggest that neither actin nor vimentin co-localize with RACK1 (Fig. 4.12A, B). In contrast, α-tubulin and RACK1 co-localize to speckle-like structures detected inside the cell (Fig. 4.12C, D).

Interestingly, while anti-tubulin antibody revealed predominantly fibrilar structure of microtubular network, it seems that brightly stained RACK1 structures co-localize with relatively brightly stained spots appearing along the microtubular fibrils, forming thus a sort of a regular RACK1-α-tubulin "nodal" network. While the nature, distribution and functional importance of such a network is not obvious, it suggests that involvement of RACK1 in Lck translocation could be mediated through its interaction with microtubular cytoskeletal network. However, whether RACK1 and microtubular network also co-localize in primary $CD4^+$ T-cells, remained to be defined.

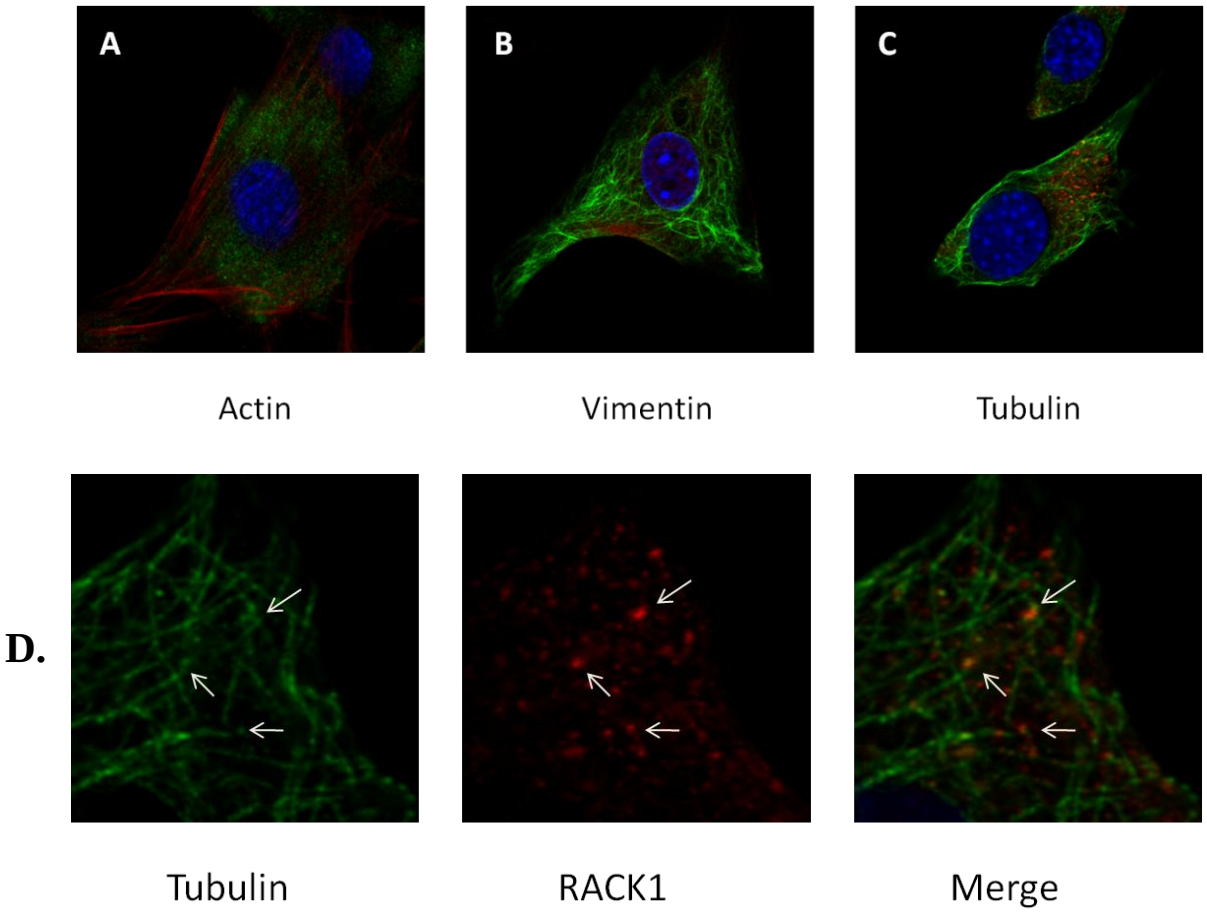


Figure 4.12 RACK1 co-localizes with tubulin but not with actin and vimentin. 3T3 cells fixed with 4% PFA were probed with **A.** phalloidine (red) and anti-RACK1 (green) **B.** anti-vimentin (green) and anti-RACK1 (red) **C.** anti-tubulin (green) and anti-RACK1 (red). **D.** Magnified image of RACK1 (red) –microtubulin (green) co-localizations. Arrows point to speckle-like structures formed by co-localized proteins. Nuclei were stained with DAPI (blue) (x400 zoom).

5 Discussion

T-cells are a prototypical example of highly sophisticated and precisely regulated signalling cells. Signalling through T-cell antigen receptor (TCR) has the potential to trigger a broad range of cellular responses. Two Src family tyrosine kinases (SFK) - Lck and Fyn - provide critical functions that predicate the generation of the most proximal signals emanating from the TCR (Filipp and Julius, 2004). It has been recently demonstrated that lipid raft (LR) plays an essential role in temporal and spatial coordination of these two kinases (Filipp et al., 2004; Filipp et al., 2003). Specifically, TCR-CD4 co-aggregation-induced Lck activation outside LR results in Lck translocation to LR where the activation of LR-resident Fyn ensues. Central in this sequence of events is the rapid translocation and subsequent enrichment of kinase active Lck in LR. While this process of Lck recruitment to LR is indispensable for T-cell activation, the mechanism underpinning this event is unknown.

The overarching goal of our investigation presented in this thesis was the characterization of the molecular mechanism, and its/their functional elements, regulating the early recruitment of signalling molecules to LR and forming immunological synapse. We addressed this task by focusing specifically on *cis*- and *trans*-acting elements regulating the process of Lck recruitment to LR. Preliminary data obtained in our laboratory (see the section 2.9) provided a compelling evidence for the existence of proteins regulating this critical aspect of Lck physiology. Notably, it pinpointed the receptor of activated C kinase (RACK1) protein as candidate molecules regulating spatial distribution and translocation of T-cell signalling kinases *in trans*. They also demonstrated the involvement of Lck C-terminal sequence (*in cis*) in targeting Lck to LR likely via tethering directly to cytoskeletal elements (Filipp et al., 2008). As targeting Lck to LR predicates enhanced TCR mediated IL-2 production (Filipp et al., 2008) and alternations in the association of Lck and other accessory molecules with LR support abnormal T-cell signalling in autoimmune diseases (Jury et al., 2004), elucidation of the nature of this process on a molecular level represents a topic of great scientific and clinical interests.

Potential members of translocation machinery were originally identified using a screening strategy based on difference in phosphorylation status of cellular proteins present in NIH3T3 fibroblast cell line expressing either a full size or C-terminal truncate

mutant (ΔFQPQP) of kinase active Y505-Lck (2-D gels, Fig. 2.7). Notably, several hypo-phosphorylated proteins derived from ΔFQPQP-Lck expressing cells and resolved on 2D gels, were recognized. It is important to emphasize that ΔFQPQP-Lck, that retains kinase activity, displayed a 60-80% reduction in their partitioning to LR (Filipp et al., 2008). So, as targeting of ΔFQPQP-Lck to LR is compromised, the hypophosphorylated proteins could be downstream targets of kinase active Lck responsible for its translocation process presumably through direct interactions with cytoskeletal elements.

One of the candidate protein selected by this screening process was identified as RACK1. Initial search for available information in public databases revealed that RACK1 fulfils the main requirement expected from the member of Lck translocation machinery: it consists of modular domains able to function as a linker or scaffold sequences for interaction with other proteins; (ii) it is a substrate for SFK Src and also can interact with Lck (Chang et al., 1998); (iii) it is involved in the intracellular translocation processes (Ron et al., 1999); and importantly (iv) is able to interact with cytoskeletal components (Osmanagic-Myers and Wiche, 2004). All these characteristics led us to investigate the ability of RACK1 to interact with Lck and the function of RACK1 in the context of Lck translocation mechanism in $CD4^+$ T-cells.

Experimental data presented in this study are the first to reveal and characterize the role of RACK1 in T-cell activation, in general, and in proximal TCR signalling, specifically. Co-expression in NIH3T3 cells of HA-tagged RACK1 with Lck variants revealed that constitutively active Y505F-Lck, but not WT-Lck, complexes with RACK1. This suggests that RACK1 preferentially binds the activated form of Lck. Structure-function analysis performed on NIH3T3 infectants ectopically expressing Y50F-Lck with its domain-inactivating mutations showed that Lck-RACK1 interaction depends on functional SH2 and SH3 domains and the C-terminal tail sequence of Lck. Importantly, Lck-RACK1 complex formation is detectable in primary $CD4^+$ lymph node T-cells and wanes shortly after activation. Remarkably, while this complex formation is induced by TCR/CD4 ligation it does not require an induction of global tyrosine phosphorylation regarded as an early biochemical marker of proximal TCR signalling.

Upon T-cell activation, only the pool of Lck molecules associated with the high molecular weight fractions (HMWFs) translocates to lipid rafts. Co-purification of

RACK1 with these fractions correlates with its possible involvement in the mobilization of Lck to LR. In addition, Lck and RACK1 co-redistribute with the same kinetics to both antibody-induced capping clusters and forming immunological synapses. This argues for the existence of a mechanism by which Lck and RACK1 are physically coupled and mechanistically transferred to these structures, perhaps by binding to some element(s) of cytoskeletal network. Recent demonstration that RACK1 is able to bind the cytoskeletal proteins (Osmanagic-Myers and Wiche, 2004) warranted an investigation into the RACK1-mediated regulatory mechanism underpinning cytoskeleton-controlled translocation of Lck to LR. In this context it is of interest that Lck was shown to be associated with cytoskeletal system (Louie et al., 1988). Our confocal microscopy data showed that RACK1 co-localizes with microtubulin component of cytoskeleton. As this is the first report of microscopically observed association between RACK1 and microtubulin, the nature and precise mechanism(s) of this co-localization is unknown at present time. Thus, in aggregate, our data strongly suggests that RACK1 is an adaptor protein involved in the regulation of Lck translocation to LR through providing a scaffold function linking Lck to the microtubular cytoskeletal network.

It is clear that while this investigation provided some answers and clues to our original questions regarding the nature, elements and mechanism of Lck mobilization to LR, it also raised many questions the answers to which are unknown at present time. We will discuss these points below.

First point relates to RACK1 domain structure and its ability to interact with Lck. It has been reported that the 6th WD40 domain of RACK1 binds directly to SH2, but not SH3 domain of Src (Chang et al., 2001). As Src and Lck share the same structural components and domain organization, we have assumed that RACK1-Lck interaction should be also dependent on SH2 domain of Lck. We have also predicted that based on the strategy of our screening protocol, the C-terminal mutant can contribute to this binding. Unexpectedly, in addition to these two domains, RACK1 was also largely abrogated with SH3-inactivated SH3 mutant of Lck. This suggests an equal importance of all three implicated domains in Lck-RACK1 binding. However, while Src-RACK1 interaction is modelled through binding of Src-SH2 domain to the tyrosine in position 246 (Y246) in 6th WD40 domain phosphorylated by Src itself (Chang et al., 2002), structural bases for RACK1 binding to SH3 and the C-terminal tail sequences, awaits

elucidation. At this time, we can only speculate that if the binding is direct, SH3 can potentially bind to proline-rich sequences present in RACK1 (Ballek and Filipp, unpublished data). If this interaction is mediated through some intermediary, it would predict a formation of multiprotein complex involving multiple protein-protein interactions. We posit that regardless of the size of this complex, involvement of SH2, SH3 and the C-terminal tail sequences of Lck will be required for its full formation and function. Clearly, more experiments have to be done to explain the involvement of all three Lck domains in the context of complex formation with RACK1. Moreover, whether the involvement of the three Lck domains also relates to its activation-induced translocation process to LR observed in primary CD4+ T-cells, remains to be determined.

As expected, transcripts of RACK1 are readily detectable in primary murine $CD4^+$ T-cells. This finding correlates with data available in the Expression profile databases (SymAtlas, http://symatlas.gnf.org), which indicates a relatively high level of RACK1 expression in $CD4^+$ and $CD8^+$ T-cells.

The second discussion point relates to the mechanism of induction of Lck-RACK1 interaction in primary $CD4^+$ T-cells. Structure-function analyses based on the co-immunoprecipitation of Lck and RACK1 from fibroblast cell line turned out to be informative. To assess whether Lck-RACK1 interaction occurs during Lck translocation to LR in early stages of proximal T-cell signalling, we adopted the protocol of antibody-mediated activation of $CD4^+$ lymph node T-cells. When this protocol is applied, Lck translocation to LR peaks at about 30 seconds after TCR-CD4 co-aggregation (Filipp, 2003).

As illustrated in Fig 4.6, complex formation between Lck and RACK1 in unmanipulated control sample was not observed. However, the most robust Lck-RACK1 immunoprecipitation signal was detected in anti-TCR+anti-CD4 pre-coated T-cells which were not stimulated by co-aggregation of these receptors. The complex formation signal gradually diminished over the time of stimulation (Fig.4.6). Interestingly, the pre-coating procedure itself did not result in increase of global tyrosine phopsphorylation (Fig.4.5, 0 sec sample). This indicates that (i) pre-coating the T-cells wiht anti-TCR and -CD4 antibodies is sufficient to induce RACK1-Lck interaction and (ii) RACK1 protein under these conditions does not become tyrosine

phosphorylated. These data, unfortunately, disallowed us to distinguish whether pre-coating of TCR or CD4 alone can induce Lck-RACK1 complex formation. However, as Lck associates with CD4, it is quite likely that the ligation of CD4 triggers subtle changes in Lck conformation not accompanied by its auto/trans phophorylation, what in turn leads to Lck-RACK1 binding. It is plausible that these conformational changes involve the release of the C-terminal tail of Lck from its 'tuck-in" conformation, making it available for interaction with RACK1. This is quite attractive model, as Lck binding to RACK1 would be initiated upon engagement of CD4 without any apparent attributes of cellular activation. In this sense, CD4 receptor would function as a mechano-receptor sensing the presence of APCs through recognition of MHCII and thus preparing T-cells for the potential receipt of full activation signal via connecting Lck with cytoskeletal network. However, it is also important to note, that translocation of Lck to LR can proceed only after coordinated engagement of CD4 and TCR to pMHC (Filipp et al., 2003). Thus, mechanism of Lck translocation to LR can be modelled as a two-step process: (i) transient engagement of CD4 receptor to MHCII during the scanning of APCs for a cognate ligand leads to "wiring" of Lck to the cytoskeletal network; this first step is independent of global increase in tyrosine phosphorylation; (ii) once simultaneous engagement of CD4 and TCR occurs, kinase active Lck is rapidly immobilize to LR and RACK1 gradually disengages from Lck.

The model suggested above is consistent with the kinetics of Lck translocation to LR: co-aggregation induced Lck enrichment in LR reaches its maximum at 30 seconds, what correlates with the maximum decrease in the amount of detectable Lck-RACK1 complexes (Filipp et al., 2003).

The third discussion point concerns the distribution of Lck and RACK1 and their co-redistribution induced upon activation of T-cells. In resting T-cells, Lck is localized to the plasma membrane (PM) whereas RACK1 is localized in the cytoplasm. A closer analysis revealed a narrow area of their overlay just underneath of PM. That suggests that Lck and RACK1 are position in a way allowing them to interact rapidly and efficiently. Upon T-cell activation, we observed a relatively rapid displacement of these proteins toward the same subcellular location characterized as "capping" structures and immunological synapses in $CD4^+$ T-cells and in $CD4^+$ T-cell:APC conjugates, respectively. Interestingly, coordinated movement of Lck and RACK1 resulted in their only marginal co-localization (Fig. 4.9). This is consistent with the fact that only 5-25%

of cellular Lck is translocated to LR after activation (Filipp et al., 2003) while the vast majority of Lck associates with soluble fractions. Further, the common pattern of re-distribution suggests that the underlying mechanism is shared by Lck and RACK1. That in turn indicates that both molecules, being associated with each other, are also attached to a common translocating device, presumably to a certain type of cytoskeletal network. RACK1 has been previously described as a carrier protein involved in the mobilization of PKCβII after activation with PMA, giving a precedence for being involved in processes of regulated intracellular translocation (Ron et al., 1999), However, as RACK1-mediated PKCβII and Lck translocation processes display distinct nature, localization, mechanism and kinetics, it would be of interest to understand how different interacting partners and surrounding cellular environment affect these distinct translocation events.

The forth point of discussion is focused on the pool of Lck subjected to LR translocation. Our data demonstrate that only HMWF-associated Lck, with the size bigger than 1000 kDa, is involved. RACK1 co-purifies with the HMWF, suggesting its association with translocation process. However, we were not able to detect RACK1 in neither the LR nor soluble fractions after SGFA, what suggests that RACK1 concentrates in pellet fraction after nuclei centrifugation. As a matter of fact, the yield of RACK1 obtained from cell lysates using 1% of NP-40 is relatively small (~10-20% of the total), indicating that RACK1 could be tightly bound to the cytoskeleton. Perhaps, detergents used for isolation of LR (Brij58, NP-40) are too mild to disrupt RACK1-cytoskeleton complexes. As their size is >1000 kDa, it suggests existence of multiprotein RACK1 complexes. Immunoprecipitation of these complexes and characterization of their components will pave the way for further advancement of this field.

And lastly, the fifth discussion point relates to the cytoskeleton as a very important part of living cells regulating most movements and translocation processes. In T-cell it has been shown that cytoskeleton plays a role in distribution of proteins within the cell membrane and is essential in formation of immunological synapse (Billadeau et al., 2007). It is mainly F-actin that regulates membrane protein movements and relocations (Viola and Gupta, 2007). Some membrane proteins capable of interaction with F-actin were identified: filamin A, ezrin, moesin) (Tavano et al., 2006). However, translocation processes dependent on F-actin and its polymerization become evident only during

formation of immature IS, several minutes after initiation of activation process. Hence, F-actin is an unlikely candidate involved in Lck translocation process which happens rapidly within 30 seconds after activation. Our attempt to identify RACK1 interacting cytoskeletal component showed co-localization pattern with microtubulin network (MTN). The signal overlay pattern forms small, speckle-like structures, indicating, that these structures likely represent some kind of vesicles or complexes, rather than growing end of microtubules. Importance of this finding is further strengthened by the fact that microtubules are associated with several distinct processes regulating T-cell function. For example, it has been shown that upon T-cell activation, Microtubule organizing centre (MTOC) rapidly reorients towards IS at T-cell:APC contact zone and microtubules serves as a railway delivering its cargo to this interface. Interestingly, cooperation of microtubules with F-actin has been also previously described and their association with LR has been suggested (Salmon et al., 2002).

Association of RACK1 with microtubulin cytoskeleton has not been previously reported and thus represent the point of our immense research interest. In addition, MTN has not been up untill now implicated in proximal phases of T-cell signalling. At present time, it is completely obscured how the interaction between MTN and RACK1 is maintained, what mechanism regulates it and what is the kinetics and dynamics of this interaction. Even though we exercise the caution in extrapolating results obtained in fibroblasts cells to CD4+ T-cells, we believe that these data provide a solid platform for initiation of studies elucidating these original findings.

Data presented in this study revealed an unappreciated complexity of mechanism controlling one of the most crucial step in proximal T-cell signalling surrounding the process of Lck translocation to LR. This process, originally studied as a mechanism controlling coordinated and sequential activation of Lck and Fyn through involvement of LR, unearthed the existence of mechanism regulating first seconds of activation of T-cells. Identification of RACK1 as the first member of membrane translocation machinery, characterizing its role as a scaffold protein linking the engagement of TCR and CD4 receptors with the process of Lck binding to MTN, opens a whole new area of research in proximal T-cell signalling.

Results presented here are in agreement with and further extend the current model of Lck translocation. They also provide further details about the molecular elements,

mechanisms and regulation of activation-induced translocation of Lck to LR. Whether this model can be extended to other signalling elements and scenarios with LR translocation phenotype, remains to be tested.

To elucidate physiological role of RACK1 in T-cell activation, we plan to generate primary T-cells where the function of RACK1 will be ablated via expression of shRNA or genetically by constructing the conditional T-cell RACK1 knock-out mice. We will also examine the involvement of microtubular cytoskeleton components by their pharmacological inhibition. It is critical to identify and characterize other members and interacting partners of RACK1 translocation machinery. From perspective point of view, detail characterization of these elements regulating targeting of Lck and other signalling molecules to lipid rafts could provide new protocols for treating immune disorders by downregulating T-cell immune responses.

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